Causes and mechanisms of red drupelet reversion in blackberries

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Statements and Declarations

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The following people and institutions contributed to the publication of work undertaken as part of this thesis:

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We the undersigned agree with the above stated “proportion of work undertaken” for each of the above published (or submitted) peer-reviewed manuscripts contributing to this thesis:

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Edgley, M., Close, D.C., Measham, P.F., 2016. The effects of nitrogen fertiliser on red drupelet disorder (reversion) in 'Ouachita' thornless blackberries. I International Symposium on Protected Cultivation in Tropical and Temperate Climates & X International Symposium on Protected Cultivation in Mild Winter Climates, Cairns, Queensland, Australia – *Oral presentation*

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Abstract

Red drupelet reversion (RDR) is a physiological disorder of blackberries, whereby individual or groups of drupelets that are black at harvest revert to red, usually after the fruit has been harvested and placed into cool storage. RDR reduces the visual and physical quality of the fruit and is considered a major physiological disorder of commercial blackberries. This thesis examined the physiochemical changes that occur during RDR development and investigated pre and postharvest factors associated with the development of the disorder.

The physiochemical properties of drupelets that were affected and unaffected by RDR were examined. The total anthocyanin concentration in black, partially red, and fully red drupelets was 1841 mg kg^{-1}, 1064 mg kg^{-1} and 769 mg kg^{-1} by fresh weight respectively. Anthocyanins containing acylated or disaccharide sugar moieties were more stable than anthocyanins with non-acylated and monosaccharide sugar moieties. The pH of partially red (3.05) and fully red drupelets (3.01) was lower than that of black drupelets (3.32). The firmness, measured by penetrometer, of partially red (1.90 N) and fully red drupelets (1.77 N) was lower than fully black drupelets (2.39 N). Electrolyte leakage over 24 h was higher from partially red (84.8 %) and fully red drupelets (90.0 %) than fully black drupelets (64.9 %). Examination by light and electron microscopy showed consistent cell disruption, separation and loss of integrity in the upper mesocarp of affected drupelets. The physiochemical symptoms associated with the development of RDR were consistent with mechanical injury, causing cell decompartmentalisation and subsequent anthocyanin degradation.

The effects of handling fruit and climatic factors at harvest on RDR incidence and severity were investigated during 10 harvests in 2017. Fruit that were handled during harvest had at least one drupelet develop RDR in 85 % of samples, while only 6 % of fruit that were not handled had any drupelets that developed the disorder.

The incidence and severity of RDR was significantly higher when fruit skin temperatures exceeded 23 °C during harvest, and these conditions were also associated with reduced skin firmness of drupelets that were affected and unaffected by RDR.

The degree of colour change following controlled, repeatable impact damage at a range of temperatures and subsequent storage conditions was measured by colourimeter. Impact injury caused a significant colour difference (ΔE) relative to the control fruit in 95 % of fruit. As temperature during impact and the subsequent rate of temperature change increased, the severity of colour change worsened.

The effects of nitrogen (N) application rate on RDR, fruit quality, and yield were investigated in a two-year trial. A high N application rate of 212 kg ha^{-1} produced fruit with significantly higher incidence and severity of RDR than medium (106 kg ha^{-1}) and low N (53 kg ha^{-1}) rates. The high N treatment increased yield through increasing the number of harvestable fruit in year one, and both the number of harvestable fruit and fruit mass in year two. Firmness and physiochemical fruit quality were not affected by N treatment.

The findings establish the major underlying physiochemical changes associated with RDR in blackberries and demonstrate the effects of abiotic factors associated with the development of the disorder in commercial settings. Future research directions and potential management techniques for reducing the incidence of RDR in commercial settings are also discussed.

Preface

Following a brief introductory and general methods chapters, this thesis is mainly composed of papers which have been published, submitted, or prepared for submission to refereed journals. Each chapter contains an explanatory preface detailing its publication status at the time of submission, its relevance to the project and thesis, and lists any relevant appendices. Each research chapter is presented with the preserved referencing style and formatting required by the targeted journal, but with the following changes:

- The numbering of headings, tables, and figures has been altered to reflect their position in the thesis.
- Where references are made to papers from this thesis, the in-text citation has been changed to the chapter number containing that paper.

The first of the chapters intended for publication is a literature review that consolidates and discusses the knowledge to date on RDR in commercial blackberries and contains some results from later chapters. The following four chapters consist of research papers, each of which addresses one or more of the aims of the project, as outlined on pages xi-xii. Following the research chapters, a general discussion, conclusions, and key recommendations of the project are presented.

Aims and structure

The broad aim of this project was to advance the knowledge of causes, mechanisms, and management practices for red drupelet reversion (RDR) in commercial blackberries. Following a review of the literature and consultation with Australian blackberry producers, four key goals were identified, and research was designed to address these:

1. *To identify and quantify the physiochemical changes occurring in drupelets affected by RDR.*

The underlying physiological mechanisms associated with RDR had not previously been reported, and so establishing this was necessary to further investigate the disorder. This involved attempting to induce RDR in blackberries and investigating the physiochemical changes occurring at a fruit, drupelet, and cellular level. This work provided a basis for understanding susceptibility to RDR and further refined the direction of the research. This aim is addressed in Chapter 4.

2. *To identify any physical or environmental factors involved in expression of RDR.*

Following the initial study identifying the physiochemical changes occurring during RDR, mechanical injury was identified as a key factor in the development of the disorder. To investigate this, multiple experiments examining the effects of handling, climatic conditions at harvest, and postharvest storage conditions on incidence and severity of RDR were undertaken. This is addressed in Chapters 5 and 7.

3. *To identify plant nutrition that may be contributing to an increase in RDR.*

An anecdotal relationship between nutrition and RDR had been observed among blackberry producers within Australia and overseas. Specifically, the hypothesis that excess nitrogen fertiliser application during harvest can significantly increase the susceptibility of blackberries to red drupelet disorder was investigated. This aim is addressed in Chapter 6.

4. *To identify and develop potential pre- or postharvest techniques to reduce the incidence of RDR.*

As well as investigating factors associated with high rates of the disorder, Chapters 5, 7, and 8 address practical techniques to reduce the incidence of RDR in commercial settings.

This thesis then concludes with the key findings, future research direction, and recommendations of the project in Chapter 8.

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Chapter 1. General introduction

This chapter includes a brief overview of the blackberry industry in Australia and worldwide, blackberry fruit taxonomy, and physiological topics that are relevant to the remainder of the thesis but are not covered in individual chapters. A review of any literature specific to RDR has been omitted for inclusion in the standalone literature review contained in Chapter 2.

1.1. Taxonomy

Blackberries are an edible summer fruit from the complex *Rubus* L. genus subgenus *Rubus* Watson (Clark and Finn, 2011), which includes a wide variety of cultivated and wild fruit crops with species found on all continents except Antarctica (Hummer, 2017). The major cultivated *Rubus* fruit include red raspberries (*Rubus idaeus* L.), black raspberries (*Rubus occidentalis* L.*)*, and blackberries, which typically do not have a species epithet because cultivated species are nearly all derived from at least two or more species (Clark and Finn, 2011).

1.2. Worldwide industry

Blackberries have historically been consumed predominantly as a wild fruit, with commercial production being a recent but fast-growing industry. Major growing regions include Serbia, the USA, Mexico, Hungary, China, and Costa Rica (Strik *et al.*, 2007). Worldwide production has grown steadily since the early 1990s, driven by factors including the need for a stable year-round supply, breeding programs allowing shipping to distant markets, and increasing consumer awareness of the health benefits of antioxidant-containing foods (Clark and Finn, 2014; Keogh *et al.*, 2010; Strik *et al.*, 2007). In 2005, 140,292 t were harvested worldwide from 20,035 ha of cultivated plantings Strik *et al.* (2007), with recent production estimated to be in excess of 25,000 ha (Clark and Finn, 2014).

1.3. Australian industry

Commercial blackberries are a minor horticultural crop in Australia, often grown in conjunction with raspberries and other small fruit. There are approximately 140 *Rubus* growers across the country in all states except the Northern Territory, producing approximately 800 tonnes of *Rubus* fruit from 613 ha, of which blackberries account for less than 10 % (Keogh *et al.*, 2010). The major production areas are the Gippsland and Dandenong Ranges in Victoria, and throughout Northern Tasmania (ARGA, 2009). The season runs from November through to May, with peak production occurring throughout January and February.

1.4. Production and harvest practices

Open field production is the predominant system used worldwide, but a growing number of producers are shifting to protected production under tunnels, shade cloth, or a combination of the two, especially for new plantings and high-value markets (Clark and Finn, 2014; Strik *et al.*, 2007). The benefits of tunnel production can vary with climate; however, tunnels generally provide a longer growing season, and canes produce more first-class fruit due to yield increases and reduced losses to pests, diseases and environmental stresses (Rodríguez *et al.*, 2012; Rom *et al.*, 2010; Thompson and Strik, 2009).

Fruit intended for fresh market consumption is recommended to be harvested directly into clamshell punnets, though it is not uncommon for producers to pick into shallow buckets and then transfer fruit to punnets in the field or pack house, particularly in areas with high labour costs such as Australia. Following harvest, fruit should be quickly forced-air cooled to 0–5 °C at 85–95 % relative humidity for storage and transport (Strik *et al.*, 2007).

Most commercial cultivars are harvested at the 'shiny black' stage of development, where shelf-life and ability to transport the fruit is best, although some cultivars retain astringency into the dull black stage and are unsuitable for export markets (Perkins-Veazie *et al.*, 1996a; Walsh *et al.*, 1983). Canes are harvested for ripe fruit every 2–5 days depending on cultivar, production system, and time of the season. Fruit is not washed nor treated prior to sale in order to reduce handling and rot incidence.

1.5. Anatomy and fruit structure

Blackberry plants are perennial, with biennial canes called primocanes in their first year of vegetative growth and, after a dormant winter period, they are known as floricanes in their second year. Floricanes produce flowers and fruit, while new vegetative primocanes are grown for the following year's crop. The first breeding programs to produce primocane-fruiting cultivars was the University of Arkansas, USA, and now additional programs are introducing additional cultivars with this fruiting habit. This fruiting habit allows fruiting during the first year and can also allow double-cropping for a second year's production (Clark and Perkins-Veazie, 2011; Clark and Salgado, 2016). Primocane cultivars were first introduced commercially in 2004 (Clark *et al*., 2005) and have had a rapid uptake among growers, particularly throughout the USA (Strik *et al.*, 2007), offering the advantages the extension of the fruiting season, the ability to double-crop, and a significant reduction in cane maintenance costs (Strik *et al.*, 2007; Thompson and Strik, 2009).

Blackberry fruit are an aggregate fruit that consist of a central torus or receptacle surrounded by a number of fleshy drupelets (Takeda, 1993). Each drupelet consists of a thin, soft epiarp, a fleshy mesocarp, and a hard endocarp (pyrene) that contains a seed. The size of the blackberry is determined by a combination of drupelet number and size, with modern cultivars producing a barrel, round, blocky, irregular or conical shape fruit weighing 8–15g (Clark and Finn, 2011).

At fruit maturity, an abscission zone forms at the base of the blackberry from the pedicle and the entire aggregate including the receptacle remains tightly together after abscission (Perkins-Veazie *et al.*, 2000). When the fruit is mature it can be easily removed from the cane with a small amount of force and will fall to the ground when overripe.

1.6. Fruit ripeness

The maturity of the blackberry fruit is typically described in a number of stages of ripeness: green, partial redness, full redness, partial or mottled black, shiny black, and dull black or overripe (Perkins-Veazie *et al.*, 2000b). The development of the red and black colour throughout the process is directly caused by the accumulation of anthocyanins in the fruit and is accompanied by an increase in size, softening, and an accumulation of carbohydrates and other nutrients.

1.7. Ripening processes

Blackberries increase in soluble sugars and decrease in titratable acidity during the ripening process. The increase in soluble sugars occurs primarily during the partial and fully black stage, with no significant increase from the shiny black to the dull black stage. Fructose and glucose are the major sugars in the fruit, existing in roughly equal amounts with negligible amounts of sucrose throughout the entire fruit development process (Kafkas *et al.*, 2006; Perkins-Veazie *et al.*, 2000b; Wrolstad *et al.*, 1980). Titratable acidity decreases approximately 50 % between the partial and shiny black stages, and 10–30 % between the shiny and dull black stages (Perkins-Veazie *et al.*, 2000b). The major organic acids vary with cultivar, but are most commonly reported as citric, malic, isocitric and lactoisocitric; with shikimic, fumaric, and succinic acid present in trace quantities (Fan-Chiang and Wrolstad, 2010; Kafkas *et al.*, 2006; Kaume *et al.*, 2012; Perkins-Veazie *et al.*, 2000b; Perkins-Veazie *et al.*, 1996a; Wrolstad *et al.*, 1980).

1.8. Development of phytochemicals

Blackberries are a rich source of phytochemicals including anthocyanins, phenolic acids, flavonols and other antioxidants that contribute to their taste, colour, aroma and nutritional profile. Wang and Lin (2000) reported on the total anthocyanin content, total phenolic content, and the oxygen radical absorbance capacity (ORAC) of various blackberry cultivars throughout three stages of ripening (green, pink, and ripe). The authors concluded that total phenolic content and ORAC values were lowest in pink berries (227–262 mg/100 g and 13.7–17.6 µmol of TE/g respectively on a wet matter basis), highest in green fruit (226–308 mg/100 g and 23.4–25.1 µmol of TE/g), and with moderate to high levels in ripe fruit (204–248 mg/100 g and 20.3–24.66 µmol of TE/g). Anthocyanins increased from 0.5–1.3 mg/100 g in green fruit to 8.8–10.6 mg/100 g in pink fruit and 133.5–171.6 mg/100 g in ripe fruit. The study also found that a linear relationship existed between total phenolic content and ORAC activity in all growth stages, as well as total anthocyanin content and ORAC activity in ripe berries. This indicated that the compounds responsible for antioxidant capacity of the fruit shifted from predominantly colourless phenols and acids at the green stage to coloured anthocyanin pigments as the fruit ripened.

1.9. Anthocyanins: biosynthesis and chemistry

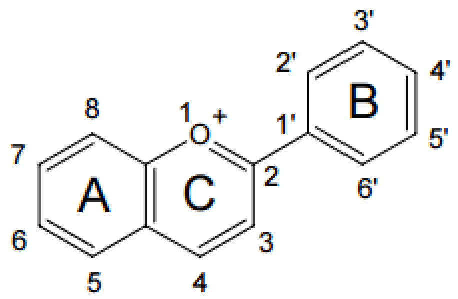
Anthocyanins, responsible for the attractive dark colour of blackberries, are water-soluble pigments belonging to a parent class of molecules called flavonoids, which are synthesised via the phenylpropanoid pathway (Cho *et al.*, 2004; Parker, 2010). These pigments can range from yellow and red to blue and dark purple depending on several factors including pH, co-pigmentation and functional groups (Welch *et al.*, 2008). They are produced by many organisms in the plant kingdom and have been observed to occur in all tissues of higher plants (Maharik *et al.*, 2009). Anthocyanins and related molecules are of significant interest to researchers and consumers due to their potential benefits for human health. Research indicates that anthocyanins and other flavonoid pigments have a wide range of biological effects including antioxidant, anti-inflammatory, antiallergenic, antiulcer, antibiotic and anti-carcinogenic properties (Cho *et al.*, 2004; Ding *et al.*, 2006; Maharik *et al.*, 2009). These properties arise from their high reactivity as hydrogen or electron donors and the ability of the polyphenol-derived radicals to stabilise and delocalise the unpaired electron, as well as their ability to chelate transition metal ions (Duan *et al.*, 2007).

Anthocyanins are biosynthesised from three molecules of malonyl CoA derived from fatty acid metabolism and one molecule of p-coumaroyl CoA synthesised from phenylalanine via the general phenylpropanoid pathway (Parker, 2010; Zhang *et al.*, 2014). Biosynthesis occurs in the cytoplasm with the major biosynthetic enzymes being located in the endoplasmic reticulum.

After synthesis, they are then transported across the tonoplast membrane into the vacuole by carrier enzymes, with glutathione-s-transferase thought to play a key role in this movement (Gomez *et al.*, 2011; Mueller and Walbot, 2001). They accumulate in the vacuole and play a number of roles in a wide range of plants including colouration to aid pollination, potential nutritional value, light absorbance and other physiological roles (Welch *et al.*, 2008).

Anthocyanidins or aglycons are the basic structures of anthocyanins, of which 23 are known to occur naturally (Castañeda-Ovando *et al.*, 2009; Welch *et al.*, 2008). These aglycons are inherently unstable and readily degrade to their corresponding aldehydes and phenolic acids or to the quinoid anhydrobase (Fleschhut *et al.*, 2006). Because of this, these molecules usually exist in nature in their glycosylated forms – anthocyanins – with sugars attached at the C3, C5, or C7 ring positions (Fig. 1-1.). Sugars found on the rings can include glucose, rhamnose, xylose, galactose, arabinose and fructose, with many anthocyanins also being acylated by aliphatic or aromatic acids (Castañeda-Ovando *et al.*, 2009; Fleschhut *et al.*, 2006; Welch *et al.*, 2008). Over 600 different anthocyanins have been identified as occurring naturally in a wide range of plants (Welch *et al.*, 2008). Anthocyanin biosynthesis is one of the most studied and well understood pathways in plant secondary metabolism (Mueller and Walbot, 2001), although the effect of these compounds on human health as well as their chemical and biochemical interactions within the human body has not been as extensively researched. The presence and stability of anthocyanins in blackberry fruit is an important factor in their visual and nutritional quality, and so maintaining the concentration of these compounds in fruit postharvest is of interest to producers and retailers.

Fig. 1-1. The basic flavylium ion structure – the backbone for anthocyanin pigments (redrawn from Pietta *et al*., 2003)



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Chapter 2. Red drupelet reversion in blackberries: A complex of genetic and environmental factors

This chapter contains a review of the current literature with reference to RDR as of January 2019. Since no comprehensive review of the literature pertaining to RDR has been published previously, the initial literature review that was undertaken has been revised to include results generated from this project, with the intention of publication. Hence, this chapter contains references to the subsequent research chapters, as well as some repetition of methodologies, results and discussion points. Conversely, some discussion points from this review are repeated briefly in later chapters, though attempts have been made to limit repetition.

This chapter has been prepared for submission for peer review pending publication of the referenced research chapters.

Abstract

Red drupelet reversion (RDR) in blackberries is a physiological disorder that causes the postharvest reddening of individual or groups of drupelets, resulting in economic loss due to a reduction in marketability. The disorder is associated with a significant reduction in anthocyanin pigment concentration, which can vary in severity causing degrees of partial or full colour change. This is associated with observations of reduced cellular structural integrity and loss of membrane integrity. Susceptibility to the disorder is heavily genotypically influenced, with an identified link between cultivar texture, postharvest weight loss and RDR incidence. Current research indicates that RDR is primarily caused by mechanical injury to the fruit that has induced cell decompartmentalisation.

This paper reviews recent advances in the understanding of RDR including the physiochemistry, causes of expression and genotypic variation in the incidence of RDR. Further study is required to clarify the mechanism for pigment degradation, and to investigate confounding genotypic and environmental effects on RDR incidence.

2.1. Introduction

Blackberries are an edible summer fruit from the complex *Rubus* L. genus subgenus *Rubus* Watson (Clark 2007). They are known for their attractive, dark colour which is due to a high concentration of anthocyanin pigments (Patras *et al.*, 2009). Blackberries are an aggregate fruit made of several smaller fruit called drupelets, which are attached to a central receptacle. The fruit have historically been harvested from wild 'brambles', with commercial production being relatively recent, supporting a rapidly growing industry (Strik *et al.*, 2007). In 2005, 140,292 t was harvested worldwide from 20,035 ha of commercially cultivated plantings, an increase of 45 % from 1995 production levels (Strik *et al.*, 2007), with recent production estimated to be in excess of 25,000 ha (Clark and Finn, 2014). The use of high tunnels for protected cropping has increased substantially in recent times (Clark and Finn 2014; Strik *et al.*, 2007), allowing an extension of the growing season, the ability to harvest in poor weather, and better fruit quality (Oliveira *et al.*, 1998; Rodríguez *et al.*, 2012; Rom *et al.*, 2010). This growth in intensive, high-value production has increased the need for crops to be free of defects and physiological disorders due to the high economic cost of losses, particularly of fruit which has been harvested, cooled, and transported.

Red drupelet reversion (RDR), sometimes referred to as red drupelet disorder, colour reversion, reddening, or red cell, is a physiological disorder of blackberries that causes individual or groups of drupelets which are black at harvest to turn red postharvest. The disorder has not been well understood until recently, with little research into the causes, mechanisms, or potential management techniques to reduce incidence. In the last decade, selection for cultivars with low incidence has become more important for breeders and producers (J Clark, personal communication, November 2018), and references to the disorder in published literature, particularly those which discuss mechanisms and causes, have become more frequent (Chapter 4; Yin, 2017; Salgado and Clark, 2016; Pérez-Pérez *et al.*, 2018).

Little published data exist regarding the extent of the financial impact of RDR on a commercial scale. It has been reported that anywhere from zero to upwards of 80 % of fruit in commercially produced cultivars are affected by the disorder (Chapters 5, 6; Clark and Moore, 2005; Clark and Perkins-Veazie, 2011; McCoy *et al.*, 2016; Salgado and Clark, 2016; Yin 2017). Pérez-Pérez *et al.* (2018) reported that 3–5 % of fruit exported from Mexico, which is the source of 95 % of internationally exported blackberries worldwide, is rejected due to RDR. In 2014, following substantial expansion of the industry over the previous decade, Mexican production was 153,000 t from 12,000 ha, most of which was exported to the United States (Pérez-Pérez *et al.*, 2018), putting the volume of rejected fruit due to RDR in the range of 4,590–7,650 tonnes for that year. Though data on rates of rejection are not available from other production areas, RDR is thought to be a major cause of loss of quality throughout North America, the United Kingdom and Australia. Current USDA marketing standards dictate that not more than 10 % of fresh blackberries by volume may lack full colour in order to classify as first grade fruit; however slight discolouration brought on by RDR is not considered a defect whilst discolouration that is bluish red to bright red is considered damage (USDA 2018). Given that most commercial cultivars have been reported to exceed 10 % discolouration under certain conditions, RDR can be considered a major encumbrance to the worldwide fresh blackberry industry.

This review consolidates the knowledge generated from previous studies and more recent focussed research into consideration of the physiochemistry, genetic variability, and abiotic factors in RDR development. Methodologies assessing for the disorder are discussed and potential directions for future research are identified.

2.2. Blackberry colour development

The maturity of the blackberry fruit is typically described in a number of stages of ripeness: green, partial redness, full redness, partial or mottled black, shiny black, and dull black or overripe (Perkins-Veazie *et al.*, 2000b). The development of the red and black colour throughout the process is directly caused by the accumulation of anthocyanins (Prasanna *et al.*, 2007; Rein, 2005), which is accompanied by an increase in size, softening, decreased acidity, and an accumulation of carbohydrates and other nutrients (Perkins-Veazie *et al.*, 2000b; Siriwoharn *et al.*, 2004b). Blackberry fruit have a relatively simple anthocyanin profile, with cyanidin-3-glucoside being the major pigment in all cultivars (Fan-Chiang and Wrolstad, 2005). Fan-Chiang and Wrolstad (2005) investigated the anthocyanin profile composition of 51 blackberry samples representing 18 different cultivars from five different geological locations over three seasons. The authors reported cyanidin-3-glucoside as making up an average of 82.9 ± 9.5 % of the total profile across the samples, with cyanidin-3-rutinoside (10.2 ± 11.5 %) and cyanidin-3-xyloside (2.5 ± 3.3 %) being the predominant minor anthocyanins. A further 10 anthocyanins have been identified in blackberries: cyanidin-3-dioxalylglucoside, cyanidin-3-malonylglucoside, cyanidin-3-arabinoside, cyanidin 3-galactoside, pelargonidin-3-glucoside, cyanidin-3-sophoroside, cyanidin-3-glucosylrutinoside, cyanidin 3-(6-malonyl)glucoside, cyanidin 3-(3-malonyl)glucoside, and malvidin 3-arabinoside (Chapter 4; Cho *et al.*, 2004; Connor *et al.*, 2005; Kaume *et al.*, 2012; Sapers *et al.*, 1986; Siriwoharn *et al.*, 2004a; Wu and Prior 2005). Quantities and ratios of the minor anthocyanins within the profile are generally not significant enough to greatly influence colour or antioxidant capacity, with only the concentration of cyanidin-3-glucoside correlating strongly with the total level of anthocyanins (Connor *et al.*, 2005). There are also significant interactions of cultivar × year and cultivar × location on the profile and concentration of anthocyanins in blackberries (Connor *et al.*, 2005).

2.3. Red drupelet reversion

2.3.1. Manifestation

Red drupelet reversion is categorised by the red discolouration of the previously black skin of ripe fruit, which can vary in severity from part of a single drupelet to multiple drupelets covering most of a fruit. Most of the colour change occurs within 24 h of the fruit entering cool storage (Chapter 7; Yin, 2017), though further development can continue in terms of the degree of colour change (Chapter 7) and the number of reverted drupelets for up to two weeks after harvest (Lawrence and Melgar, 2018). In Chapters 4 and 7 we demonstrated that the CIELAB colour coordinates, lightness

(L*) and chroma (C*), of affected drupelets were higher than the control drupelets, and that the CIELAB colour parameters of affected drupelets could vary enough to produce a significant colour difference (ΔE).

Development of RDR has been linked to mechanical injury to fruit incurred during harvest and transport. Pérez-Pérez *et al.* (2018) reported that vibration in storage significantly increased RDR incidence over control fruit, with incidence increasing with the length of vibration treatment. In Chapter 5 we demonstrated that handling fruit during harvest was associated with development of RDR in 85 % of fruit compared with only 6 % of fruit which was not handled during harvest.

Empirical observation of RDR indicates that manifestation of the disorder is associated with mechanical injury, but that confounding genotypic and abiotic factors affect the incidence and severity of the disorder, which is discussed hereafter in this review.

Fig. 2-1. Red drupelet reversion on 'Ouachita' blackberry fruit in punnets

2.3.2. History of incidence and evaluation

Morris *et al.* (1981) discussed the discolouration of hand and machine-harvested blackberries for processing. The authors reported significant effects of harvest method, harvest temperature, and holding time on pureed raw and canned samples, though the discolouration of drupelets on fresh fruit was not addressed. Incidence of RDR in fresh fruit was first documented by researchers at the USDA and the University of Arkansas in the early 1990s (Clark, 2015; Perkins-Veazie and Clark, 2011; Perkins-Veazie *et al.*, 1996b). The development of a standard protocol for postharvest performance evaluation by researchers from these institutions included an assessment for RDR development (Clark, 2015), though at the time breeding programs were more focussed on resistance to mould, leakiness, and softening, so reversion was not a high priority when selecting new cultivars (Perkins-Veazie and Clark, 2011). It was noted that susceptibility to the disorder is strongly genotypically influenced (Clark and Finn, 2011; Perkins-Veazie *et al.*, 1996b), and though cultivars with low incidence of RDR could be bred for, several seasons of postharvest evaluation of potential cultivars were needed due to the significant variation in incidence between contrasting seasons (Clark and Finn, 2011).

Various methodologies have been used in the assessment of fruit for incidence and severity of RDR, which presents difficulties when comparing results across studies and is important to note when assessing a cultivar for susceptibility to the disorder based on previous research. The first published data on the incidence of the disorder used the protocol developed by researchers at the USDA and the University of Arkansas (Perkins-Veazie *et al.*, 1996b). The protocol rated fruit on a yes/no scale for the presence of RDR after seven days of storage at 2 ± 0.5 °C and 95 % relative humidity. For a fruit to be categorised as having RDR, a cluster of three or more berry drupelets had to be red, and these numbers were then converted to a percentage based on the total number of fruit with RDR in a harvested clamshell punnet. This protocol continued to be used in subsequent years spanning more than a decade, and data collected using this or similar methodology has been reported in various studies (Clark, 2013; Clark *et al.*, 2014; Clark and Perkins-Veazie, 2011; Perkins-Veazie *et al.*, 1996b). In 2012, RDR was of growing importance to producers and researchers, and since then methodologies have been altered to reflect this (J Clark, personal communication, November 2018). In various studies from 2013 onwards, authors using the yes/no scale have used a lower incidence level of a single red drupelet on a fruit to be classified as having RDR (Clark *et al.*, 2014; McCoy *et al.*, 2016; Salgado and Clark, 2016). Salgado and Clark (2016) and Salgado (2015) reported both the percentage of fruit with RDR and the number of fruit with 0, 1–3, or 4+ affected drupelets, classifying fruit with 1–3 red drupelets as 'low to mid' and fruit with 4+ red drupelets as 'high' levels of RDR. This allowed for greater accuracy in assessing cultivar susceptibility to the disorder than a yes/no scale. Segantini *et al.* (2017) and Yin (2017) counted the total number of drupelets on each fruit and used the number of reverted drupelets per fruit to calculate the percentage of total drupelets with RDR. Lawrence and Melgar (2018) also used a yes/no scale for the presence of RDR but used an incidence level of five 'completely red' drupelets for a fruit to be classified as having reversion.

Chapters 4-7 used methodology that attempted to address both the number of reverted drupelets per fruit as well as the severity to which each drupelet was affected. For this, drupelets were classified as being 'fully black' (FB), 'partially red' (PR), or 'fully red' (FR). The authors then reported the 'Red Drupelet Index' per fruit, which was a number calculated by the formula:

$$RDI = number\ of\ PR\ drupelets + (2 \times number\ of\ FR\ drupelets)$$

A higher RDI score reflected the impact of reverted drupelets on fruit visual quality, as drupelets with more red flesh would impact fruit quality proportionally.

All of the above-described methodologies involve subjective interpretation of what constitutes a reverted drupelet. Because of this, comparing rates of RDR incidence and severity between studies with different methodologies and potentially varying thresholds for what constitutes a reverted drupelet may be flawed. To address this issue and speed up assessment, Worthington *et al.* (2017) developed a system which used digital image analysis to rapidly and accurately count the incidence of reversion across several cultivars. The authors reported significant correlations between the digital image analysis and manually counted subjective data for both percent reverted drupelets and percent reverted berries. The use of imaging software may remove bias or ambiguity involved in subjective ratings, speed up reversion assessment, as well as reduce difficulty comparing data sets where different methodology has been applied. We consider this to be a logical approach to pursue in future studies to ensure objectivity and repeatability, though it may not be practical for smaller studies, and it is unclear if this approach can account for differences in the degree of colour change. Technologies such as imaging techniques which allow for rapid assessment of RDR incidence may be particularly useful in commercial pack houses for quality control purposes.

Regardless of the methodology used for future research, care should be taken when comparing new data to previous work. For repeatability and comparability, it would be pertinent to report multiple levels of reversion incidence. For example, where authors choose to count drupelets per fruit expressing RDR, the inclusion of the percentage of fruit with 1+, 3+, and 5+ red drupelets in presented data (Chapter 5) would allow easier comparison to other studies, along with building a larger body of knowledge for RDR incidence among cultivars and environments.

2.3.3. Impact of RDR on objective and consumer perceived fruit quality

RDR is not thought to alter the taste of blackberries significantly and causes no significant changes to the total soluble sugar content or acidity, although affected drupelets have a slightly lower pH (3.07) than black drupelets (3.43) from the same fruit (Chapter 4) that presumably is not detected by consumers, though no studies have investigated this. Chapter 4 demonstrates a significant reduction in firmness measured by penetrometer on affected drupelets, indicating a degradation of textural quality (Kader, 2002) and potential postharvest shelf-life implications. Soft blackberries have more decay and leakiness than firmer fruit (Payasi *et al.*, 2009; Perkins-Veazie *et al.*, 1997), so the reduction in firmness associated with reversion is thought to have further implications on shelf-life. Furthermore, firmness is a key quality factor in consumer perception of quality (Ross *et al.*, 2009) and softening is often associated with loss of freshness (Redgwell and Fischer, 2002).

Research has shown that RDR reduces the visual quality of fruit. In an online consumer survey of 879 North American blackberry consumers, 72.9 % of consumers preferred punnets with no RDR and 20.1 % of consumers preferred punnets with low incidence compared to punnets with high incidence (Dunteman *et al.*, 2019). The red colour of reverted ripe fruit was associated by consumers to unripe fruit (Dunteman *et al.*, 2019).

2.3.4. Physiochemistry

The colour change of RDR is associated with a decline in anthocyanin pigment concentration, which has been demonstrated across multiple cultivars and environments (Chapter 4; Morris *et al.*, 1981; Pérez-Pérez *et al.*, 2018; Perkins-Veazie and Collins, 1993). Perkins-Veazie and Collins (1993) examined the anthocyanin content of ‘Arapaho’, ‘Navaho’, ‘Chester’, ‘Cherokee’, ‘Choctaw’, and ‘Shawnee’ berries, reporting that as the percentage of fruit with RDR increased, anthocyanin concentration decreased. Pérez-Pérez *et al.* (2018) reported that in ‘Tupy’ fruit, reverted drupelets contained 58.5 % of the concentration of monomeric anthocyanins relative to non-reverted drupelets (4.5 and 7.7 mg cyanidin-3-glucoside g^{-1} freeze dried sample, respectively). In Chapter 4 we analysed the full anthocyanin profile of black, partially red, and fully red ‘Ouachita’ drupelets, reporting that partially reverted and fully reverted drupelets contained 57.8 % (1064 mg kg^{-1} FW) and 41.7 % (769 mg kg^{-1} FW) of the total anthocyanins of black drupelets (1841 mg kg^{-1} FW), respectively. As well as this, the latter study found that anthocyanins containing disaccharide or acylated sugar moieties were less readily degraded in affected drupelets than anthocyanins containing monosaccharide sugar moieties.

Significant cellular structural changes have been observed in drupelets affected by the disorder. Pérez-Pérez *et al.* (2018) demonstrated that in ‘Tupy’ fruit, drupelets without symptoms of RDR showed greater cellular integrity and order relative to reverted drupelets. Chapter 4 reported that reverted drupelets contained more visible intercellular spaces, less uniform cells and fewer ruptured cells than control drupelets. This was accompanied by an increase in electrolyte leakage, an indicator of cell plasma membrane damage (Bajji *et al.*, 2002), and reduced pH. Both Chapter 4 and Pérez-Pérez *et al.* (2018) discussed the link of cell damage with the demonstrated reduction in anthocyanin concentration and change of colour. Salgado and Clark (2016) identified differences at a cellular level between cultivars with high and low susceptibility to the disorder. The authors demonstrated that 'crispy' cultivars, which present high firmness, low weight loss, and low susceptibility to the disorder, had thicker cell walls, increased cell density and increased cellular integrity relative to 'non-crispy' cultivars with moderate to high susceptibility to RDR.

2.3.5. Impact of fruit maturity

It has been noted that fruit which is shiny black at harvest is more prone to developing RDR than fruit harvested dull black, and that no relationship appears to exist between drupelets that leak juice and RDR (Perkins-Veazie and Clark, 2011; Perkins-Veazie *et al.*, 1996b). Blackberries are known to undergo significant physiological changes from the shiny black to dull black ripeness stages including a reduction in internal turgor pressure, reduced firmness, a reduction in total acidity, increased anthocyanin and phenolic concentration, elevated rates of ethylene production and a decrease in the activity of oxygen-scavenging enzymes (Brummel, 2006; Burdon and Sexton, 1993; Perkins-Veazie *et al.*, 2000b; Perkins-Veazie and Collins, 1993; Siriwoharn *et al.*, 2004a; Wang and Jiao, 2001). An explicit explanation for the reduction in susceptibility to RDR between the shiny and dull black stages has not been established as yet; however, it is likely that one or more of the significant physiochemical alterations between these ripeness stages are involved in the change. Turgor pressure and firmness can act independently on fruit bruise susceptibility (Baritelle and Hyde, 2001; Van Zeebroeck *et al.*, 2007), with a reduction in turgor pressure often being associated more strongly with decreased bruise susceptibility than an increase in firmness (Baritelle and Hyde, 2001; Garcia *et al.*, 1995). Fruit at the shiny black stage may be more susceptible to injury due to higher turgor pressure, which reduces when fruit reaches the dull black stage (Brummel, 2006).

2.3.6. Genotypic variation in RDR susceptibility

A substantial genotypic influence on susceptibility to RDR has been a consistent observation in all studies to date that have assessed incidence of the disorder in two or more cultivars. Table 2-1 demonstrates the variance between cultivars as reported by the current literature.

Table 2-1. Variance in cultivar susceptibility as described by cited studies[1]

Cultivar	1+ red drupelets (%)	3+ red drupelets (%)	5+ red drupelets (%)	% total affected drupelets	RDI[2]	Source(s)
Apache		0.0–4.7	32–49			13, 13, 19, 42
Arapaho		0.0–6.1	44–51			13, 42
BlackMagic™/APF-77	72.9	15.3–66.1				13
Choctaw		47.5				60
Chester Thornless		0–10	19–34			42, 61
Cheyenne		41.3				60
Crispy genotypes	13.2					73
Natchez	43.0–72.0	5.0–66.6	49–66	1.0		13, 19, 42, 45, 77, 90, 92
Navaho		0.0–18.8	9–14			42, 60, 61
Non-crispy genotypes	41.0					73
Osage	19.3–40.7	1.0–18.4	30–40	1.7		13, 32, 45, 77, 90, 92
Ouachita	18.8–85.0	0.0–81.0	30–40	6.1	0.08–5.4	13, 28, 29, 30, 42, 45, 63, 90, 92
Prime-Ark® Traveler	17.8–45.3	9.0	19–49			16, 45
Prime-Ark®45/APF-45	16.8–45.4	0.0–25.3		1.6		19, 42, 45, 77, 92
Prime-Jan®/APF-8		4.7				19
Prime-Jim®/APF-12		75.3				19
Shawnee		56.3				60
Triple Crown			66–73			42
Tupy	26.3–60.7	5.2–31.5				13, 19
Von			45–71			42

[1] Values for control fruit at the shiny black ripeness stage are reported where available. Only minimum and maximum values across all studies for each cultivar are reported.
[2] Red drupelet index calculated by formula discussed in section 2.3.2.

'Crispy' cultivars (from the University of Arkansas breeding program) are categorised by their distinct texture as opposed to the melting texture of regular cultivars. Genotypes expressing the crispy trait have demonstrated very low susceptibility to RDR, improved cell wall integrity, a reduction in cell separation, increased firmness, and reduced postharvest weight loss (McCoy *et al.*, 2016; Salgado and Clark, 2016; Yin 2017). Fruit textural properties with regard to softening depend heavily on the dissolution of the middle lamella, reduction of cell-to-cell adhesion, and the weakening of parenchyma (Brummel, 2006; Paniagua *et al.*, 2014; Redgwell *et al.*, 1997) driven by the degradation of pectins and hemi-celluloses (Huber, 1983; 1984).

In crisp fruit, downregulation of genes relating to polygalacturonase, a group of enzymes largely responsible for controlling softening through pectin solubilisation, results in firmer fruit (Hadfield and Bennett, 1998; Paniagua *et al.*, 2014). As fruit susceptibility to mechanical injury is strongly linked with firmness (Moggia *et al.*, 2017), the reduced susceptibility of these cultivars to developing RDR may be associated with a reduction in cell disruption caused by mechanical injuries (McCoy *et al.*, 2016; Salgado and Clark, 2016). Given that moisture loss is a major cause of reduced postharvest firmness in other berry fruit (Nunes *et al.*, 1995; Paniagua *et al.*, 2013), reduced moisture loss may also lead to reduced RDR incidence.

The influence of the cultivar ripening period on cultivar susceptibility has not been investigated, though this may be a factor in cultivar susceptibility because it will determine the abiotic stresses a cultivar is likely to experience during ripening. It is possible that, given the reported effects of climatic conditions during harvest on RDR development (Chapter 5; Lawrence and Melgar, 2018; McCoy *et al.*, 2016; Yin, 2017), cultivars which ripen during cooler periods may be less susceptible to the disorder, though this will vary with climate, cane management and production system.

The tacit evidence outlined in this section indicates that greater cell wall integrity, underpinned by intact pectins, may explain the major distinction in RDR susceptibility between ‘crispy’ and ‘non-crispy’ cultivars.

2.3.7. Intra-seasonal variation

It has been colloquially suggested that the time of season may play a role in fruit susceptibility to the disorder (Perkins-Veazie and Clark, 2011), though no studies have examined any abiotic or physiological explanation for this. Perkins-Veazie *et al.* (1996b) found that early season fruit had higher numbers of fruit with RDR than late season fruit for six cultivars. In Chapter 6 we reported that early season fruit had higher rates of incidence and severity in 2016, but no clear seasonal trend existed in 2017.

In Chapter 6 we also reported trends for declining fruit firmness and fruit size over the season for both years, although no correlation was identified between individual fruit firmness and RDR susceptibility. Temperature at the time of harvest was a significant factor in RDR incidence in the second year, a result which was consistent with other studies (Chapter 5; McCoy *et al.*, 2016; Morris *et al.*, 1981; Yin, 2017). Lawrence and Melgar (2018) reported harvest date as a significant factor in reversion incidence across 10 cultivars and suggested that the associated environmental factors at each harvest date were the contributing factor in this variation.

Seasonal variations in fruit quality and susceptibility to various physiological disorders have been noted for a range of other horticultural crops. Bollen (2005) reported that early season apples (*Malus* × *domestica* Borkh.) are often more susceptible to bruise damage than late season fruit; a trend which has also been reported in various other stone and pome fruit (Crisosto *et al.*, 2001; Lu Arpaia *et al.*, 1987). This is often attributed to fruit firmness, though the physiology behind this has not been clearly outlined. Opara (2007) suggested that this trend in apples could have several contributing factors including fruit curvature, shape, crop load and irrigation scheduling. Given the demonstrated trends for blackberry fruit size, firmness and seasonality, (Chapter 6; Fernandez-Salvador *et al.*, 2015), and those between fruit firmness and RDR (Salgado and Clark, 2016; Yin, 2017), it is likely that seasonality can play a role in RDR incidence. However, with confounding climatic variables also varying with seasonality, it may require substantial data to fully explore any trends; and no studies have thoroughly explored this. Fruit chemistry can also vary within season: Ali (2012) found that sugars, acidity, ellagic acid, and ascorbic acid content of blackberries all varied within seasons, and ellagic acid, a major phenolic acid in blackberries (Huang *et al.*, 2012), was significantly higher in early season fruit while ascorbic acid was higher in late season fruit. It is unclear what role if any, the fruit's physiochemical qualities may play in RDR, though it is worth noting that these factors may further convolute any intra-seasonal trends.

2.3.8. Inter-seasonal variation

Significant variation in the incidence of RDR between seasons of fruit from the same plants has been noted by multiple authors (Chapter 6; Clark, 2013; Clark *et al.*, 2014; Clark and Perkins-Veazie, 2011; Perkins-Veazie *et al.*, 1996b). No study has examined this in detail, though suggestions have been made regarding contrasting abiotic stresses (Chapters 5, 6). An example of inter-seasonal variation can be seen in the substantial increase in RDR incidence across multiple cultivars at the same location in 2008 and, to a lesser extent, 2009 reported by Clark *et al.*, 2014 and Clark and Perkins-Veazie, 2011. Though seasonal climatic trends have not been addressed in depth regarding any effect on RDR, authors noted that the 2008 and 2009 seasons were particularly wet (Clark and Perkins-Veazie, 2011). This observation is confirmed by available climate data, with records showing very high values of extreme precipitation events for Arkansas during these years (Runkle and Kunkle, 2017). It is possible that the variation between seasons may also be confounded by differences in the climatic conditions at individual harvests; therefore, more data are needed to identify inter-seasonal trends which may impact RDR incidence.

2.3.9. Climatic factors

Development of RDR appears to be strongly linked to pre- and postharvest climatic and environmental factors including temperature, relative humidity and plant water status. In particular, increased fruit temperatures have been associated with a higher incidence and severity of RDR by various studies. McCoy *et al.* (2016), Yin (2017), and Chapter 5 all reported relatively consistent trends when investigating the role of harvest time on RDR; fruit harvested at times associated with skin temperatures exceeding roughly 23 °C generally had a higher incidence and severity of the disorder. In contrast, Lawrence and Melgar (2018) found no correlation between air temperature during harvest and RDR, though a lower variance in harvest times and associated temperatures was used in this study than in the trials that reported an effect.

It has been suggested that increased temperature during harvest may cause fruit to be more susceptible to mechanical injuries incurred by handling and transport, resulting in cell decompartmentalisation (Chapter 5; McCoy *et al.*, 2016; Yin, 2017). Though this effect appears to vary with cultivar (McCoy *et al.*, 2016; Yin 2017), increased susceptibility to mechanical injury at warmer temperatures, particularly when caused by compression, has been observed in other berry fruit (Ferreira *et al.*, 2009; Nunez-Barrios *et al.*, 2005). This effect has been linked to the decreased firmness and associated decrease in failure stress of fruit at warmer temperatures (Ahmadi *et al.*, 2010; Nunez-Barrios *et al.*, 2005), as well as the increased metabolic response of fruit to damage as temperature increases (Van Linden *et al.*, 2006).

In conclusion, although largely correlational, there is strong circumstantial evidence for the effect of abiotic conditions on the incidence of RDR, particularly given the general finding of 23 °C being a critical temperature threshold reported across cultivars, growing systems and hemispheres. Further study is warranted to clarify confounding factors such as genotypic variation and the influence of other abiotic factors on RDR development.

2.3.10. Nutritional factors

In Chapter 6 we conducted a two-year trial investigating the effects of nitrogen fertiliser application rates on RDR and postharvest quality in commercial, tunnel-grown 'Ouachita' fruit. In this trial an application rate of 212 kg N ha^{-1} significantly increased RDR relative to 106 and 53 kg ha^{-1}. No other studies have investigated any nutritional influences on RDR incidence; however, it has been anecdotally suggested by growers that overapplication of both nitrogen and potassium may tend to increase RDR incidence.

2.3.11. Postharvest factors

Postharvest storage conditions have been observed to affect RDR, though effects appear to vary with cultivar, and results are inconsistent across studies. Perkins-Veazie *et al.* (1996b) found that holding fruit at 20 °C overnight, before transferring to 2 °C, reduced RDR incidence by 1–10 % of fruit relative to fruit stored at 2 °C immediately following harvest for six cultivars. Lawrence and Melgar (2018) investigated the effects of a 90-minute delay to cool storage at ambient outside shade temperature on RDR incidence, reporting that the delay in cooling resulted in a significant reduction in RDR for 'Apache', an increase in 'Von', and no significant effect for eight other cultivars. This inconsistency may be due to methodology that involved multiple harvest dates with air temperatures ranging from 18–27 °C between cultivars, which could obscure any effects of cooling rate on RDR development.

In Chapter 7 we reported that fruit which undergoes rapid temperature changes from over 25 °C to a core temperature of less than 2 °C developed a more significant severity of RDR, measured by changes to CIELAB colour coordinates, than fruit which is cooled at a slower rate. The rate of temperature change affecting physiological bruise response is a phenomenon that has been noted in other crops; bruising has been shown to alter fruit thermal properties through altering transpiration rate, density, conductivity, and consequently heat production and dissipation through affected tissue (Segovia-Bravo *et al.*, 2011; Van Linden *et al.*, 2003). Given the link between RDR and fresh weight loss (Yin, 2017), it is plausible that damaged flesh is prone to increased transpiration rate, giving a physiological explanation for the increased susceptibility to rapid temperature changes postharvest. While a delay to postharvest cooling may be beneficial in some situations, this approach should be treated with caution, given that even a short delay of two hours to cooling has been shown to negatively affect the shelf-life of *Rubus* fruit (Robbins and Moore, 1992).

Storage at excessively low temperatures has also been discussed as a factor influencing susceptibility to the disorder, with anecdotal suggestions that storage at 7–9 °C reduces incidence compared to

0 °C. McCoy *et al.* (2016) observed no clear trends between storage at 1 °C and 5 °C for seven days in fruit from four harvests, but suggested further investigation with larger trial sizes was warranted, as the unusually wet season may have influenced results. Temperatures approaching 0 °C have been shown to alter berry and stone fruit mechanical properties through decreasing elasticity, making cells more prone to failure from impacts (Crisosto *et al.*, 1993; DeMartino *et al.*, 2002; Ferreira *et al.*, 2009; Lidster and Tung, 1980).

Evidence suggests that temperature at the time of and following mechanical stress to fruit affects the physiological response to RDR development. Temperatures exceeding 23 °C have been associated with increased susceptibility in a range of conditions (Chapter 5; McCoy *et al.*, 2016; Yin, 2017), and rapid temperature changes have been suggested to intensify the colour change (Chapter 7). Further study is needed to fully explain the physiological mechanisms responsible for these relationships, as this is one area that provides opportunities to develop practical management techniques to reduce susceptibility to RDR.

2.4. Other disorders

Various other physiological disorders of blackberries present similar symptoms to RDR, which are often confused with RDR, particularly when occurring concurrently. RDR is thought to be independent of these (Chapter 4; Perkins-Veazie and Clark, 2011), though detailed examination of any interrelations between RDR and other disorders have not been reported on in the literature.

2.4.1. Redberry disease

Red berry mite (*Acalitus essigi*) is a serious pest in commercial blackberries, causing what is commonly known as ‘redberry disease’ (Davies *et al.*, 2001; Scott *et al.*, 2008). The mite feeds on developing blackberry fruit, beginning at the green fruit stage and significantly increasing during the red fruit stage (Davies *et al.*, 2001). This results in uneven ripening of the fruit, with the affected drupelets surrounding the cortex remaining red, hard, and inedible, whilst the base of the fruit develops normally, but nevertheless causes the fruit to be unsaleable.

2.4.2. Uneven ripening

Some cultivars are also prone to uneven ripening with similar symptoms to redberry disease, yet with a seemingly physiological cause. In these cases, the drupelets at the base of the fruit, which in redberry disease will not progress from the hard-red ripeness stage, ripen at a slower rate than the drupelets around the apex, resulting in half of the fruit being dull black and half being shiny black. Very little is known regarding the causes of this, with the only brief mention in the previous literature by Alford (1979), who distinguished this disorder from redberry disease caused by redberry mites.

2.4.3. White drupelet disorder

Blackberries and raspberries are prone to developing tan to white discolouration on drupelets, known as white drupelet disorder (WDD) (Stafne *et al.*, 2017), and thought to be caused primarily by UV radiation (Bolda, 2009). Increases in WDD are seen in association with rapid increases in temperature combined with decreased humidity, resulting in a sudden increase in UV radiation exposure to developing fruit (Bolda, 2009). Increased WDD has also been observed with rainfall followed by sunshine (J Clark personal communication March 2019). Rain, overhead irrigation and misting, and shade cloth to reduce UV have all been recommended to reduce incidence of WDD (Spiers *et al.*, 2014; Stafne *et al.*, 2017). Quezada *et al.* (2007) found that WDD increased in 'Heritage' red raspberries with an increase in nitrogen fertigation rates.

2.5. Gaps in knowledge and future research

Research focussed on RDR has increased substantially over the last decade, though significant knowledge gaps still exist which warrant further study. The chemical mechanism for anthocyanin degradation, while thought to be associated with cell decompartmentalisation induced by mechanical injuries (Chapter 4; Pérez-Pérez *et al.*, 2018; Salgado and Clark, 2016) remains unclear.

The development of crispy cultivars which are resistant to RDR is promising. It appears likely that this resistance is linked to increased firmness and reduced moisture loss due to the unique texture of these cultivars, though further research is needed to confirm these theories.

The impact of warm temperatures during handling appear to be consistent across multiple cultivars and environments, with increasing temperature being associated with higher rates and severity of RDR. It is likely that this relationship is also linked to changes in membrane fluidity and associated susceptibility to mechanical injury, though additional data is needed to confirm this.

Research into techniques available to reduce the disorder is lacking. Current recommended practices involve manipulating picking practices to reduce double handling and picking at times associated with cooler temperatures (Chapter 5; McCoy *et al.*, 2016; Perkins-Veazie and Clark, 2011; Yin, 2017). Utilising shade cloth, canopy architecture or other external structures that encourage shaded fruit may help by directly reducing fruit temperature.

Opportunities exist to explore other agronomic and directed management techniques to address RDR. Preharvest calcium sprays have previously been shown to be effective in increasing blackberry firmness (Aguilar Ayala *et al.*, 1992; Morris *et al.*, 1980), which may increase resistance to mechanical injury associated with RDR development. These studies were done on older cultivars however, and newer cultivars may have similar or increased enhancement. The established link to N fertiliser application rate (Chapter 6) presents options for agronomic management of the disorder through manipulating fertiliser regimes, though the effects of nutrient availability on fruit quality in *Rubus* production often vary significantly with environmental factors (Strik, 2008).

In terms of methodology for assessing the incidence and severity of the disorder, future studies should be aware of the variation used in previous studies when reviewing literature, designing experiments, interpreting data and presenting results. Any assessments using subjective rating systems should be done by well-trained staff, and preferably one or a small number of people over the course of a study.

In general, 24 h in cold storage appears to be enough time for the development of colour change, though some further development may occur for up to two weeks postharvest.

Given the current evidence suggesting temperature during and after handling blackberries can significantly influence RDR incidence and severity, further study is warranted into the effects of storage temperature and RDR susceptibility.

2.6. Conclusions

Red drupelet reversion is a major physiological disorder in blackberries that is responsible for significant wastage and economic loss in the commercial sector. The colour change is associated with a decrease in anthocyanin concentration, reduced cellular integrity, reduced drupelet firmness and lower pH. The disorder is genotypically influenced, with evidence that cultivar firmness, cell wall formation and weight loss can influence cultivar susceptibility. Abiotic stresses, particularly warm temperatures during harvest, have been linked to high rates of the disorder through increasing the mechanical injuries incurred during harvest and handling.

Substantial variation exists in the methodologies used throughout the previous literature for assessing RDR incidence and severity. As well as varying methodologies, further confounding factors such as subjectivity of assessment and unreported abiotic factors present difficulties in comparing rates of expression between climates and cultivars. High firmness, crispy texture, and reduced fresh weight loss appear to contribute to reduced cultivar susceptibility to the disorder. Further research is needed to investigate confounding and possibly interactive genotypic, abiotic, nutritional and postharvest factors in the development of RDR.

2.7. Acknowledgments

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Chapter 3. General materials and methods

This chapter contains additional information pertaining to the location of trials undertaken in this project, as well as further information on cultivar choice and statistical analysis used.

3.1. Location of Field Trials

Field trials and fruit harvesting for all published work was undertaken at Costa Berries Dunorlan farm site, Dunorlan, Tasmania, Australia (41.5 °S, 146.6 °E). The region has a cool temperate climate and is a notable area for *Rubus* production, with rapid expansion of small fruit production in the area over the last decade. The region has a mean yearly rainfall of 995 mm, peaking in the winter months, which receives roughly double that of the summer months (Fig. 3-1).

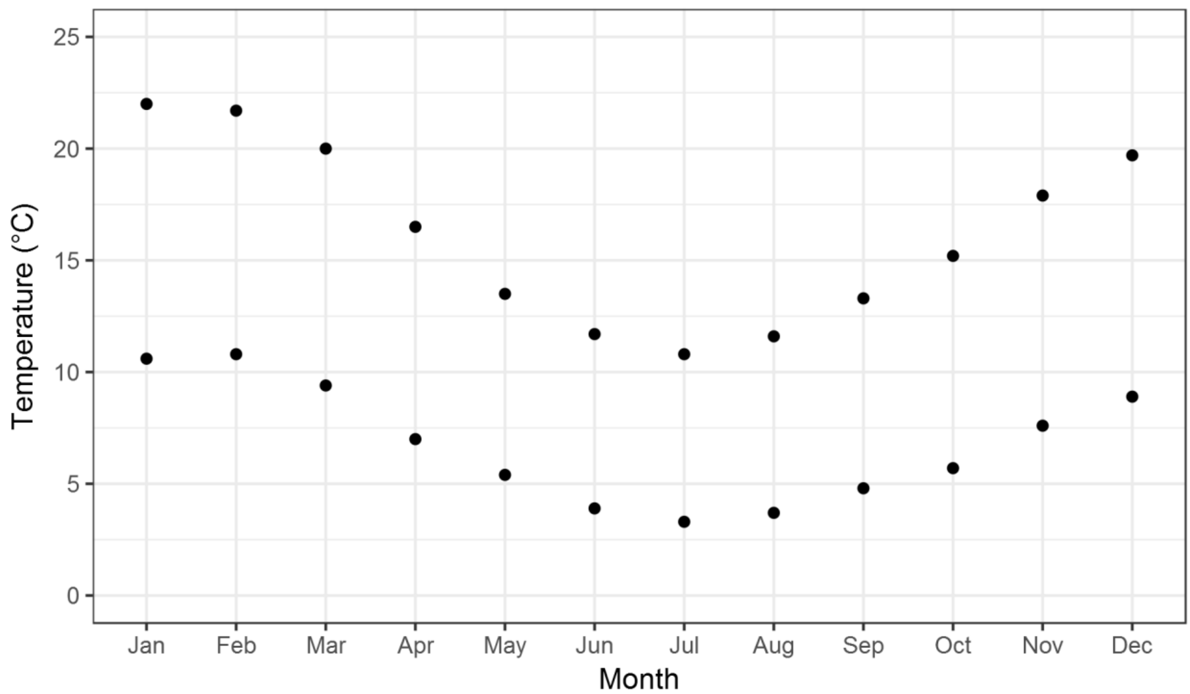

Fig. 3-1. Mean monthly minimum and maximum temperatures (°C) over the last 20 years at the Dunorlan field site. Data sourced from the Australian Bureau of Meteorology.

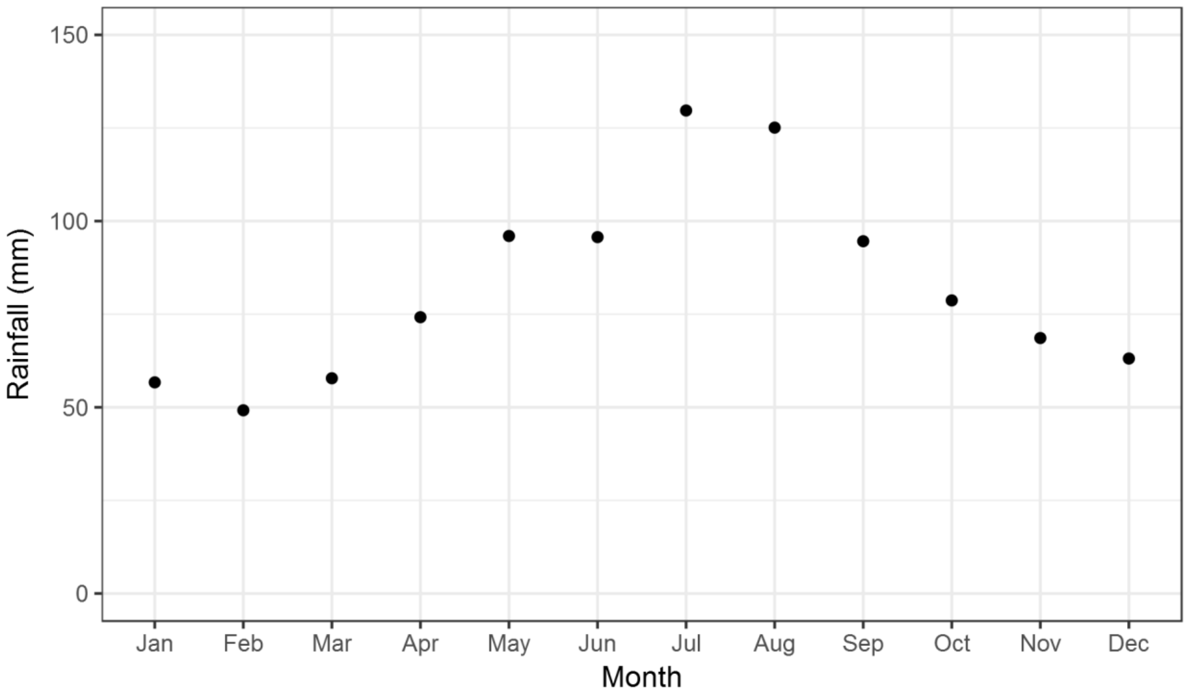

Fig. 3-2. Mean monthly rainfall (mm) over the last 20 years at the Dunorlan field site. Data sourced from the Australian Bureau of Meteorology.

Some preliminary fieldwork was also carried out at Westerway Raspberry Farm, Westerway, Tasmania, Australia (42.7 °S, 146.8 °E). This site is in the Derwent Valley region, has a cool temperate climate and is a notable area for the production and processing of fresh market berry fruit. Data from these preliminary field harvests were not published; however, they were used to guide the planning of larger trials and informed the methodology used.

Fig. 3-3. Map of Tasmania with the Dunorlan (A), Westerway (B), and Tasmanian Institute of Agriculture (C) sites labelled.

3.2. Cultivar selection

The cultivar 'Ouachita' was selected for the majority of the experimental work for a number of reasons: the cultivar historically produces good quality fruit for a long season at the Dunorlan field site, it is the predominant cultivar grown in the state, the fruit produced has a medium-to-high susceptibility to red drupelet disorder in Australia, and has relatively small incidence of other pests and diseases, and the block on which the cultivar is grown is flat with uniform soil and little wind exposure.

The cultivar is erect and thornless, producing blocky, conical fruit, which is non-uniform in shape. The cultivar was produced by the University of Arkansas blackberry breeding program, and when first released it produced fruit with an average weight of 5.8 g (Clark and Moore 2005). Fruit produced at the experimental site over the experimental period had a mean weight of 9.9 g over the three years of study (Chapters 4–7).

Some preliminary work was first undertaken on ‘Navaho’ and ‘Loch Ness’ fruit harvested from both sites.

3.3. Statistical analysis

R (R Core Team, 2017) versions 3.3.0 or later was used for all the statistical analysis undertaken. Specific statistical tests and R packages are described in the relevant experimental chapters. Unless otherwise stated, a significance level of $P<0.05$ was used for all statistical analysis. All graphs were generated using the package ‘ggplot2’ (Wickham, 2009).

Chapter 4. Physiochemistry of blackberries (*Rubus* L. subgenus *Rubus* Watson) affected by red drupelet reversion

This chapter addresses the first key goal of the project: to identify and quantify the underlying physiochemical associated with RDR development. This work was necessary to provide a fundamental base of knowledge for the remainder of this research as well as future study in this field.

This chapter has been reviewed and accepted, pending minor revisions, to Postharvest Biology and Technology.

Abstract

Red drupelet reversion (RDR) is a physiological disorder causing individual or groups of drupelets on blackberries that are black at harvest to turn red during postharvest cool storage. The objectives of this study were to examine and quantify the physiochemical changes occurring in flesh affected by RDR. Drupelets were classified as 'fully black', 'partially red', or 'fully red'. The total anthocyanin concentration in black, partially and fully red drupelets was 1,841 mg kg^{-1}, 1,064 mg kg^{-1} and 769 mg kg^{-1} fresh weight respectively. Anthocyanins containing acylated or disaccharide sugar moieties were more stable than anthocyanins with non-acylated and monosaccharide sugar moieties. The pH of partially red (3.05) and fully red drupelets (3.01) was lower than black drupelets (3.32). Firmness of partially red (1.90 N) and fully red drupelets (1.77 N) was lower than that of fully black drupelets (2.39 N). Examination by light and electron microscopy showed cell disruption, separation and loss of integrity in the upper mesocarp of affected drupelets. Electrolyte leakage over 24 h was significantly higher from partially red (84.8 %) and fully red (90.0 %) than fully black drupelets (64.9 %). The data are consistent with RDR in blackberries arising from mechanical injury that causes cell decompartmentalisation and subsequent anthocyanin degradation.

Keywords: Anthocyanin; cell disruption; firmness; electrolyte leakage

4.1. Introduction

Red drupelet reversion (RDR) is a physiological disorder in blackberries causing drupelets that are black at harvest to revert to red, usually during postharvest cool storage, which can seriously detract from the appearance of fruit (USDA, 2018). The disorder is not well understood, but has been the subject of increasing interest coinciding with the significant growth in blackberry production since the early 2000s (Strik *et al.*, 2007). Recent studies have linked the disorder to bruising caused by vibration damage during transport (Pérez-Pérez *et al.*, 2018) and handling during harvest (Chapter 5). McCoy *et al.* (2016), Yin (2017) and Chapter 5 demonstrated that harvest times associated with warmer conditions coincide with increased rates and severity of RDR. It has been reported that the disorder may be genotypically and environmentally influenced (Clark and Finn, 2011; Perkins-Veazie and Clark, 2011), and fruit firmness has been linked to cultivar susceptibility (Salgado and Clark, 2016). Salgado and Clark (2016) observed cellular structural differences between 'crispy' and 'non-crispy' cultivars and suggested that the disorder may result from physical damage to the fruit. The underlying physiochemical changes in fruit affected by RDR, and the mechanisms causing these changes have not been examined. This study aims to investigate and consider the physiochemistry that occurs in blackberry flesh affected by RDR.

4.2. Materials and methods

4.2.1. Plant material and site description

Fruit (*Rubus* spp. *cv '*Ouachita') were harvested throughout January and February 2016 and 2017 from a commercial berry farm located in Dunorlan, Tasmania, Australia (41.5 °S, 146.6 °E) every 15 days throughout January and February 2016 and 2017 for eight total harvests. Plants were grown under high tunnels covered in UV-transmitting polythene and subject to standard commercial agronomic management practices for erect type blackberries (Strik and Finn, 2012; Strik et al., 2007). Distance between plants was 1 m and distance between rows was 2.5 m for erect type blackberries (Strik and Finn, 2012; Strik et al., 2007). Distance between plants was 1 m and distance between rows was 2.5 m.

Fruit at the shiny black ripeness stage were hand-harvested into 125 g punnets in mid-morning and were transported on ice to the Tasmanian Institute of Agriculture, Hobart for storage at 4 °C and 90 % relative humidity until analysis or freezing. Fruit was transferred to storage within 3 h of harvest, and unless otherwise stated, all fruit assessments or dissection and freezing were undertaken within 30 h of harvest on room temperature fruit. For analyses suitable for frozen samples (titratable acidity, pH, soluble sugars, total anthocyanins) individual drupelets for each sample were carefully removed from the receptacle of the fruit with a scalpel, weighed on a Mettler Toledo Scientific Balance, placed into plastic test tubes with lids and frozen at -80 °C for later analysis.

4.2.2. Red drupelet reversion assessment

Three degrees of RDR were assigned to individual drupelets in this trial. 'Fully Black' (FB) drupelets were completely unaffected by RDR. 'Partially red' (PR) drupelets had some part but not all visible skin affected by RDR, with drupelets selected for further analysis having approximately 50 % of the visible surface affected. 'Fully red' (FR) drupelets had all visible flesh a distinct red colour. All fruit assessed was at the 'shiny black' stage of development, and drupelets chosen for further analysis were not affected by any other insect or pathogen damage.

4.2.3. Anthocyanin content and profile

Fully Black, PR, and FR drupelet samples frozen at -80 °C were thawed to 4 °C overnight in a refrigerator then combined at a 1:10 ratio with methanol containing 0.01 % HCl (v/v) and homogenised in a Retsch Grindomix GM 200 (Retsch, Germany) for one min. The homogenised sample was subjected to ultrasonic treatment in darkness for 45 min to aid anthocyanin extraction. Samples were then centrifuged at 4,000 g and 4 °C for 45 min to obtain a clarified extract. The supernatant was removed, and the extraction process was repeated twice before combining the supernatants. The solvent was then removed by rotary evaporation at 35 °C. The dried anthocyanin extracts were then made up to 25 mL with HPLC-grade water for LC-MS analysis.

Samples were analysed using a Waters Acquity H-Class UPLC instrument coupled in series to a Waters Acquity Photo Diode Array detector and a Waters Xevo triple quadrupole mass spectrometer. A Waters Acquity UPLC BEH C18 column (2.1 mm × 100 mm × 1.7 µm) was used. The mobile phase consisted of two solvents: 5 % (v/v) Formic acid in water (solvent A) and Acetonitrile (solvent B). The UPLC program was initially 95 % A held for 2 min, followed by a linear gradient to 92.6 % A at 4.0 min and 85.1 % A at 7 min, which was held for 2 min before returning to initial conditions and re-equilibration for 3 min. The flow rate was 0.35 mL min^{-1}, the column was held at 35 °C, and the sample compartment was at 6 °C.

The mass spectrometer was operated in positive ion electrospray mode with a needle voltage of 2.6 kV, a desolvation gas (Nitrogen) flow of 950 L hr^{-1} at 450 °C and a cone gas flow of 50 L hr^{-1}. Anthocyanins were identified by multiple reaction monitoring.

Photo diode array detection was enabled over the range 210 to 500 nm and specifically at 497 nm. Anthocyanins were quantitated using a five-point external calibration curve at 497 nm using the standards cyanidin-3-glucoside, cyanidin-3-rutinoside and cyanidin-3-xyloside. Other anthocyanins were expressed as cyanidin-3-glucoside equivalents.

Standards of cyanidin-3-glucoside, cyanidin-3-rutinoside, and cyanidin-3-xyloside were obtained from Sapphire Bioscience (Redfern, New South Wales, Australia). All other chemicals were sourced from Imbros, Hobart, Australia.

4.2.4. Colour change

CIELAB colour space values (L*, a*, b*) were measured using a CR-400 colorimeter (Konica Minolta, Australia). Chroma (C*) and hue angle (h^o) were calculated by the formulas:

$$C^* = \sqrt{(a^* \times a^*) + (b^* \times b^*)}$$

$$h^\circ = tg^{-1}(\frac{b^*}{a^*})$$

4.2.5. Electrolyte leakage

Individual drupelets were carefully excised from the receptacle using a scalpel to detach the drupelet without damaging it. Drupelets were weighed, and 12 replicates of five drupelets of each colour were selected for each replicate so that the samples were within 5 % of of the same total mass, so drupelets with similar flesh to mass ratios were assessed. Drupelets were carefully washed three times after excision with distilled water to remove surface electrolytes before being placed in 30 mL of distilled deionised water and gently shaken to encourage dissipation of solutes in a 20 °C water bath for 24 h. The electrical conductivity (EC) of the solution (EC_1) was measured using a HI8733 Multi-range EC Meter (Hanna Instruments, Australia) and the vessels were autoclaved at 120 °C for 20 min. Samples were then shaken again in a 20 °C water bath for 24 h before a final electrical conductivity measurement was taken (EC_2). Electrolyte leakage was calculated by the formula shown below and expressed as the percentage of total electrolytes leaked over the initial 24 h period.

$$Electrolyte\ leakage\ (\%) = (EC_1/EC_2) \times 100$$

4.2.6. Physiochemical properties

Five replicates of 30 drupelets per colour (FB, PR, FR) were excised from fresh blackberry fruit. Upon thawing to room temperature, samples were homogenised with a mortar and pestle, then centrifuged at 4000 g for 15 min to obtain a clarified extract. Total soluble solids (TSS) were measured as ^{o}Brix using a Shibuya Optical hand-held refractometer. Titratable acidity (TA) and pH were measured using a Metrohm 702 SM Titrino automated titrator. A 5 mL sample of homogenate was mixed with 15 mL distilled water and titrated with 0.1 N NaOH until the turning point. Titratable acidity was expressed as percent citric acid equivalent (g L^{-1}).

4.2.7. Firmness

Drupelet firmness was measured using a GUUS Fruit Texture Analyser (GUUS, South Africa) equipped with a 2 mm diameter flat bottomed probe set to descend at 25 mm s^{-1} with a minimum resistance force of 0.03 kg and a test depth of 2 mm. Whole fruit (n = 50) were cut in half length-wise to provide a flat bottom surface and FB, PR and FR drupelets were centred under the probe for testing. A total of 50 individual drupelets of each colour were measured. All fruit had a skin temperature of 5 ± 1 °C at the time of analysis, measured using a HI99556 infrared thermometer (Hanna Instruments, Australia).

4.2.8. Microstructural and ultrastructural observations

For observations at a fruit and drupelet scale, fruit were examined using a Leica M80 Stereo Microscope (Leica, Wetzlar, Germany). Fruit were dissected as needed using a scalpel to view cross-sections and drupelet-receptacle connections.

For ultrastructural observations, five whole FB and FR red drupelets were washed three times with 2.5 % (v/v) glutaraldehyde in 0.1 M phosphate buffer and then fixed in this solution under vacuum (five days total). Samples were then dehydrated in an acetone series of 20 % increasing increments beginning at 20 % with three changes of 100 % acetone. The drupelets were infiltrated with Spurr's resin (ProSciTech, Brisbane, Australia) in 25 % increasing increments beginning at 25 %, and embedded in 100 % Spurr's resin at 70 °C overnight. Semi-thick sections (4-5 µm) were cut with a glass knife fitted to a Reichert Om U2 ultramicrome. The sections were transferred to a drop of sterile distilled water and gently heat fixed. Slides were stained with 1 % (w/v) toluidine blue O, rinsed with 1 % (w/v) sodium borate solution, rinsed in distilled water, decolourised in 70 % ethanol, rinsed again in distilled water and air dried. Sections were mounted in Euparal (Australian Entomological Supplies, New South Wales, Australia) beneath a cover slip and heat cured. Slides were examined using a Leica DM1000 Compound Microscope.

Images from both microscopes used were taken using a Leica DMC6200 at manually adjusted heights for a range of focal points and processed using Leica Application Suite. Images from a range of focal points were then digitally 'z-stacked' together using CombineZP image processing software (Hadley 2010) to extend the image's depth of field.

Surface structures were viewed at high magnification using a FEI MLA650 environmental scanning electron microscope (ESEM) at the Central Science Laboratory, University of Tasmania, Hobart, Australia. Three samples of FB and FR were dissected from whole fruit, mounted and viewed under low vacuum (130 Pa), using an acceleration voltage of 20.0 kV and a working distance of 1–30 mm.

4.2.9. Statistical analysis

Structural anatomical observations were made comparing side-by-side images from replicates of drupelets. Numerical data were subject to analysis of variance (ANOVA), with means compared using Tukey's Honest Significant Difference (HSD) tests. R version 3.5.1 (R Core Team 2018) or later was used for all analyses.

4.3. Results

4.3.1. Anthocyanin content and profile

The content of all identified anthocyanins in FB, PR, and FR drupelets are listed in Table 4-1. Total anthocyanin content was significantly lower in FR compared to FB drupelets ($P<0.01$). PR drupelets were not significantly different to FB ($P = 0.054$) or FR ($P = 0.59$) drupelets. Cyanidin-3-glucoside was the dominant anthocyanin making up an average of 89 % of the profile of FB drupelets. Three other anthocyanins – cyanidin-3-xyloside, cyanidin-3-dioxalyl-glucoside and cyanidin-3-(6''-malonylglucoside) made up a combined 11.1 % of the profile. Cyanidin-3-arabinoside, cyanidin-3-rutinoside, cyanidin-3-(3''-malonylglucoside), and pelargonidin-3-glucoside all were minor anthocyanins within the profile, combining to less than 0.5 % of the total anthocyanin content.

Table 4-1. Anthocyanin content and profile in FB, PR and FR drupelets.

Anthocyanin	Anthocyanin concentration (mg kg^{-1} FW)		
	FB	**PR**	**FR**
Cyanidin-3-glucoside	1633.3 ± 528.3 ^{a}	929.6 ± 493.7 ^{b}	658.6 ± 135.0 ^{b}
Cyanidin-3-xyloside	95.4 ± 24.4 ^{a}	55.6 ± 27.8 ^{b}	40.5 ± 7.7 ^{b}
Cyanidin-3-(6''-malonylglucoside)	53.2 ± 10.6	45.2 ± 23.2	35.7 ± 7.9
Cyanidin-3-dioxaloylglucoside	47.7 ± 11.6	34.6 ± 14.5	31.6 ± 6.7
Pelargonidin-3-glucoside	2.0 ± 0.9 ^{a}	0.9 ± 0.4 ^{b}	0.7 ± 0.2 ^{b}
Cyanidin-3-(3''-malonylglucoside)	1.9 ± 0.4 ^{a}	1.2 ± 0.6 ^{ab}	0.9 ± 0.4 ^{b}
Cyanidin-3-arabinoside	1.2 ± 0.4 ^{a}	0.7 ± 0.4 ^{ab}	0.5 ± 0.1^{b}
Cyanidin-3-rutinoside	0.6 ± 0.4	0.4 ± 0.2	0.3 ± 0.5
Total	1840.6 ± 572.7 ^{a}	1064.1 ± 553.5 ^{ab}	768.9 ± 154.3 ^{b}

Means followed by different letters in each row are different at $P<0.05$
Values are mean ± standard deviation (n=5)

Pelargonidin-3-glucoside was proportionally the most readily degraded anthocyanin within the profile in fruit affected by RDR, with an average decrease of 66 % between FB and FR flesh. Cyanidin-3-glucoside was degraded more readily than any of the other cyanidin anthocyanins with an average 59.7 % reduction between FB and FR flesh.

Cyanadin-3-arabinoside and cyanidin-3-xyloside were degraded in ratios consistent with the average within the profile from FB to FR (57.5 %) (Table 4-2), resulting in no significant change in their contribution to the total profile in RDR affected flesh. Cyanidin-3-(3''-malonylglucoside) significantly degraded in FR relative to FB drupelets, but by a lesser amount (52.3 %), resulting in a small increase in the proportion of this anthocyanin within the profile of FR drupelets. Cyanidin-3-rutinoside, cyanidin-3-dioxaloylglucoside, and cyanidin-3-(6''-malonylglucoside), which are all di-glucosides, were all not significantly different between FB and FR drupelets, causing their respective proportional concentrations within the profile to increase; however, the concentrations of cyanidin-3-rutinoside varied considerably between samples. All the trends described for the ratio of degradation of anthocyanins within the profile from FB to FR were consistent, but with more variation between FB and PR drupelets.

Table 4-2. Percentage of the total anthocyanin profile per individual anthocyanin.

Anthocyanin	Percentage (%) of total profile		
	FB	**PR**	**FR**
Cyanidin-3-glucoside	88.80	87.36	85.65
Cyanidin-3-xyloside	5.20	5.22	5.27
Cyanidin-3-(6''-malonylglucoside)	3.11	3.87	4.66
Cyanidin-3-dioxaloylglucoside	2.60	3.50	4.11
Pelargonidin-3-glucoside	0.11	0.08	0.09
Cyanidin-3-(3''-malonylglucoside)	0.10	0.11	0.12
Cyanidin-3-arabinoside	0.06	0.07	0.07
Cyanidin-3-rutinoside	0.03	0.04	0.04

4.3.2. Colour change

CIELAB colour parameters are reported in Table 4-3. Lightness (L*), redness (a*) and chroma were both significantly different between all three classifications of RDR incidence. The hue of FR was lower than FB drupelets.

Table 4-3. CIELAB colour space of FB, PR, and FR drupelets.

Parameter	FB	PR	FR
L*	16.5 ± 3.3^{a}	19.2 ± 1.1^{b}	25.3 ± 3.3^{c}
a*	1.8 ± 0.8^{a}	11 ± 1.2^{b}	17.4 ± 2.5^{c}
b*	0.8 ± 0.5^{b}	4.1 ± 1.0^{b}	5.1 ± 1.0^{b}
Chroma	2.0 ± 0.3^{a}	11.7 ± 2.9^{b}	18.3 ± 3.3^{c}
Hue^{o}	24 ± 4.4^{a}	20.4 ± 8.3^{ab}	16.3 ± 3.6^{b}

Means followed by different letters are different at P<0.05.
Values are means ± standard deviation (n=50).

4.3.3. Electrolyte leakage

The total percentage of electrolytes leaked into solution after 24 h increased significantly (P<0.05) from FB to PR and FR drupelets, but PR and FR were not significantly different (P = 0.24) (Fig. 4-1). FB had the highest amount of variance in total leakage with an average of 64.9 ± 10.4 %, whilst PR and FR drupelets leaked 84.8 ± 4.1 % and 90.0 ± 1.9 % of their total electrolytes respectively.

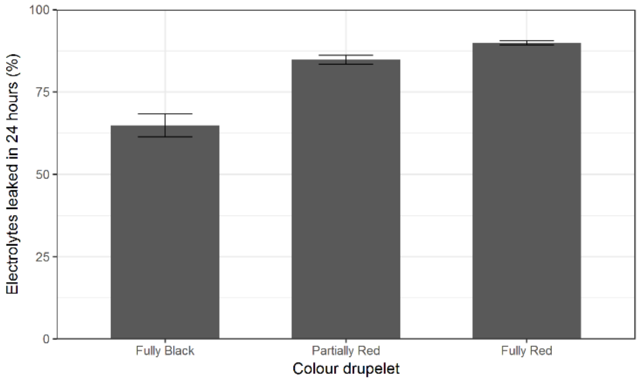

Fig. 4-1. Percent of total electrolyte leakage over 24 h in distilled water of FB, PR, and FR drupelets. Error bars show one standard deviation. n = 12.

4.3.4. Fruit chemical quality

The pH of FB drupelets was significantly higher than FR drupelets, and PR drupelets were not significantly different to FR or FB (Table 4-4). There were no significant differences in TSS or TA between colour drupelets.

Table 4-4. Quality characteristics of FB, PR, and FR drupelets.

Drupelet colour	**FB**	**PR**	**FR**
Titratable acidity (g/L)	1.07 ± 0.6	1.01 ± 0.3	1.03 ± 0.3
Soluble augars (^{o}Brix)	10.5 ± 1.1	10.3 ± 1.2	10.4 ± 1.5
pH	3.31 ± 0.2 ^{a}	3.05 ± 0.1 ^{ab}	3.01 ± 0.1 ^{b}

Means followed by different letters in each row are different at P<0.05
Values are mean ± standard deviation (n=20)

4.3.5. Firmness

Drupelet firmness measured by penetrometer is shown in Fig. 4-2. FB drupelets were significantly firmer than both PR and FR drupelets (P<0.05), but PR and FR were not different (P=0.24).

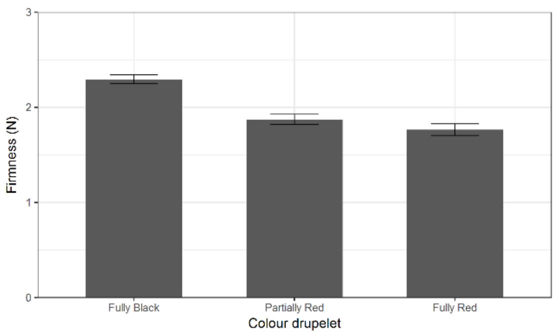

Fig. 4-2. Firmness (N) of FB, PR, and FR drupelets. Error bars show one standard deviation. n = 50.

4.3.6. Macrostructural observations

Skin of drupelets affected by RDR was visibly red, and often less taut with a sunken, wrinkled, or damaged appearance (Fig. 4-3). Remnants of broken pistils were observed on affected drupelets but were intact on FB drupelets.

Fig. 4-3. Micrographs of FB drupelets (A) and drupelets affected by RDR (B).

Dissection of drupelets showed that RDR affected the both the internal flesh making up the mesocarp of drupelets as well as the epicarp (not pictured).

4.3.7. Ultrastructural observations

The cells making up the epicarp of the blackberries were visible as a single layer of smaller, thicker-walled uniform cells relative to the fleshy mesocarp that is made up of larger, less-uniformly sized cells (Fig. 4-4, 4-5). FB drupelets contained little or no ruptured cells (Fig. 4-4A), whilst rupturing was prevalent in all replicates of FR drupelets (Fig. 4-4B), particularly in the mesocarp. The thicker-walled cells making up the epicarp were less-often ruptured than those making up the mesocarp, and no visible tears in the epicarp were observed.

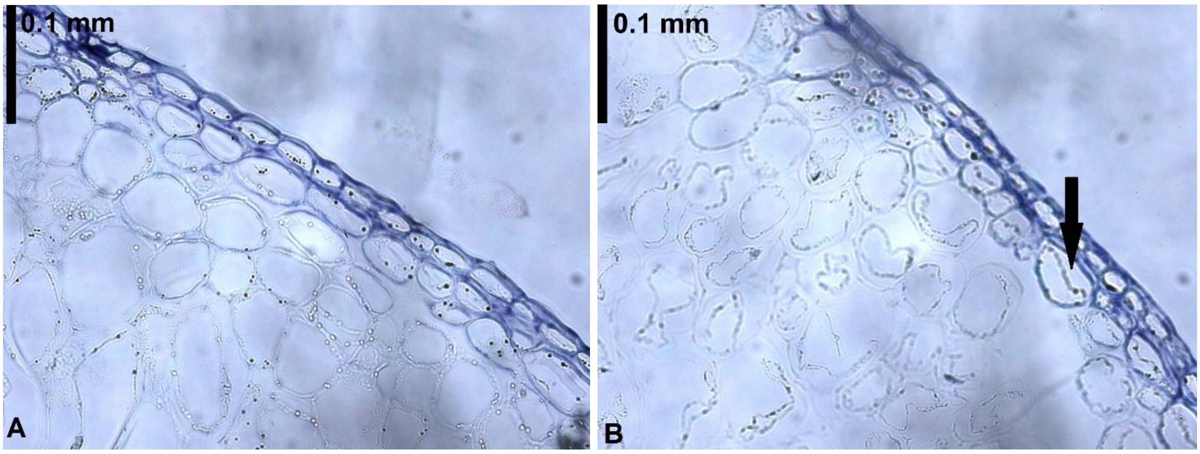

Fig. 4-4. Optical micrograph of FB (A) and FR (B) drupelets. Arrow indicates damaged cell.

The upper mesocarp of FB drupelets contained uniform cells and no intercellular spaces or tears were observed in any replicates. The same area in FR cells contained prevalent, large intracellular spaces (Fig. 4-5B) where cell-to-cell adhesion was reduced.

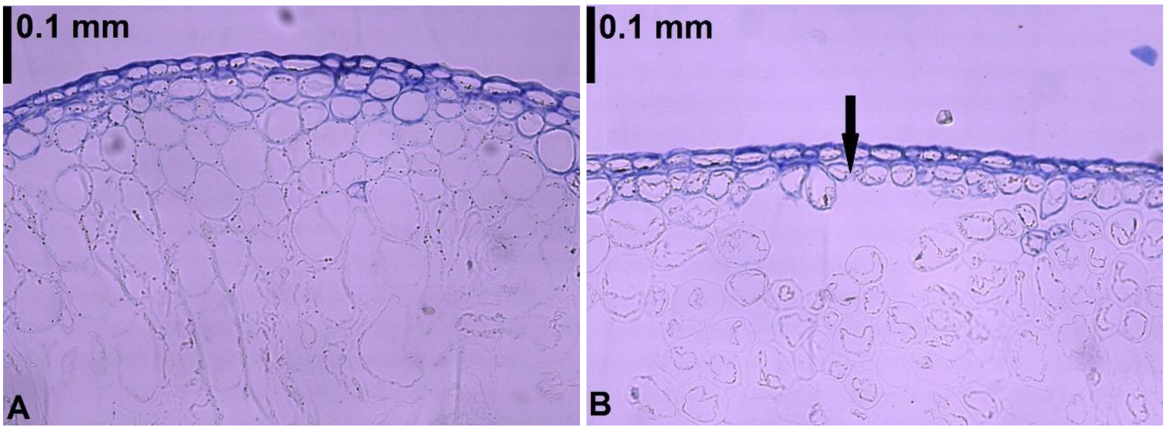

Fig. 4-5. Optical micrograph of a FB (A) and FR (B) drupelets. Arrow indicates intercellular space. Intercellular spaces within three layers of cells were visible in seven out of nine FR replicates. No FB sections contained visible intracellular spaces in this area.

4.3.8. ESEM

The epicarp offresh FB and FR drupelets was observed at high magnification by ESEM (Fig. 4-6). No significant tears or breaks in the fruit epicarpwere observed in either colour drupelet. FB drupelets appeared smooth whilst the FR drupelets had a noticeably undulated surface.

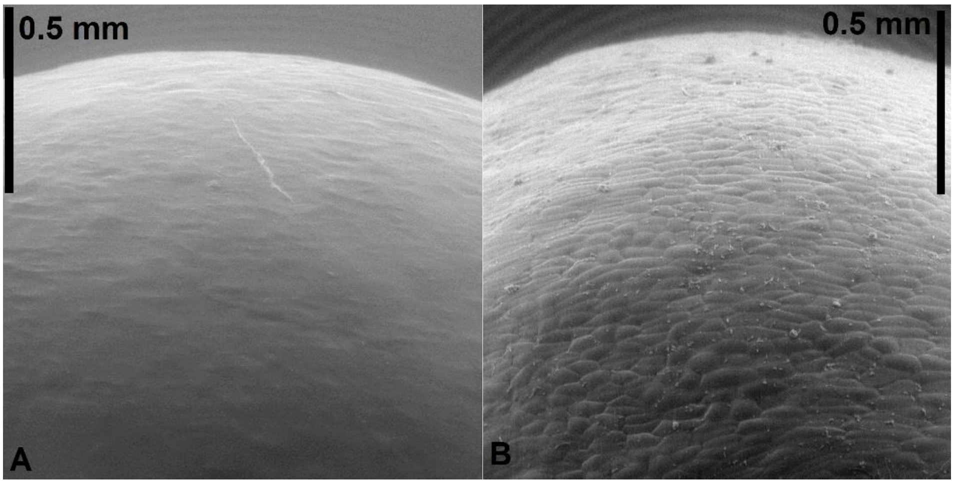

Fig. 4-6. FB (A) and FR (B) drupelets under ESEM. Undulations were notable on all replicates (n=3) of FR and PR drupelets, whilst all FB drupelets had smooth surfaces with few discernible features.

4.4. Discussion

4.4.1. Anthocyanin content, profile, and colour change

The significant reduction in total anthocyanin concentration between FB and FR drupelets (1,840.6 and 768.9 mg kg^{-1} fresh weight respectively) indicates that the colour change seen in drupelets affected by RDR is caused by a degradation of the anthocyanins within the affected tissue (Table 4-2). Though the difference between FB and PR drupelets was marginally insignificant (P = 0.054), variation was high and so a larger sample size may have shown a significant decline in the total anthocyanin content for PR drupelets (1,064.1 mg kg^{-1} fresh weight). The average concentration of total anthocyanins in FB drupelets is consistent with previous reports for fresh and frozen 'Ouachita' blackberry fruit (Kim *et al.*, 2016; Pantelidis *et al.*, 2007; Perkins-Veazie *et al.*, 2000b; Sebesta, 2014; Wang and Lin, 2000). All of the individual anthocyanins identified have been reported as present in various blackberry cultivars previously (Chen *et al.*, 2012; Fan-Chiang and Wrolstad, 2005; Kaume *et al.*, 2012; Siriwoharn *et al.*, 2004b).

Our data demonstrate that different anthocyanin species are distinctly affected by the process leading to RDR. The concentration of pelargonidin-3-glucoside, the only non-cyanidin anthocyanin identified, was more significantly reduced than all cyanidin anthocyanins identified (P<0.05). The acylated pigments, cyanidin-3-dioxaloylglucoside and cyanidin-3-(6''-malonylglucoside), did not significantly differ between FB and FR drupelets; and the concentration of cyanidin-3-(3''-malonylglucoside) was relatively less reduced than non-acylated anthocyanins ($P<0.05$). This variation in the response of individual anthocyanins to RDR resulted in a slightly different proportional makeup of the full anthocyanin profile in FR flesh compared to FB flesh (Table 4-3), however, cyanidin-3-glucoside was still the dominant pigment. The structure of the anthocyanidin backbone as well as the type of and extent of glycosylation and methoxylation of the aglycone can determine how readily an anthocyanin is degraded (Garcia-Viguera and Bridle, 1999; Patras *et al.*, 2010; Rein, 2005). Hydroxylation of the aglycone can stabilise anthocyanidins, though this effect does not always correlate consistently with increased stability to the anthocyanin itself (Rein, 2005). Cabrita *et al.* (2000) investigated the stability of common anthocyanidin-3-glucosides in aqueous solutions and reported that peonidin-3-glucoside was more stable than the more hydroxylated petunidin-3-glucoside, but that delphinidin-3-glucoside was less stable than the more hydroxylated cyanidin-3-glucoside. Our data indicates that the more substituted cyanidin-based pigments were more stable against RDR than pelargonidin, albeit only one pelargonidin pigment existed in the profile and in relatively small amounts.

The structure of the sugars and other functional groups attached to the anthocyanidin is another factor which can influence the stability of the anthocyanin (Welch *et al.*, 2008; Zhao *et al.*, 2017). Acylation has been reported to stabilise anthocyanins as well as decrease the sensitivity to changes in pH (Bąkowska-Barczak, 2005; Escribano-Bailón *et al.*, 2004; Kırca *et al.*, 2007; Zhao *et al.*, 2017). Our data are consistent with this, with two of three acylated anthocyanins not decreasing significantly in concentration.

The mechanism behind this stabilisation effect may be contributed to by a reduction in polarity (da Costa *et al.*, 2000), steric hindrance (Mazza and Brouillard, 1990), or co-pigmentation resulting from acyl groups (Brouillard, 1981; Figueiredo *et al.*, 1998; Zhao *et al.*, 2017).

Though the variation in results for the concentration of cyanidin-3-rutinoside was high, the lack of a significant difference between FB and FR drupelets may have been due to this anthocyanin being a disaccharide, as opposed to the monosaccharides, which were all significantly different, as the larger sugar moiety may protect the anthocyanin from nucleophilic cleavage. (Brønnum-Hansen and Flink, 1985; Rubinskiene *et al.*, 2005; Xiong *et al.*, 2006).

The three degrees of RDR incidence assigned to drupelets all had significantly different CIELAB colour profiles (Table 4-1). It is well established that anthocyanins are the main contributors to the vibrant colours of berry fruit, and these data are consistent with previous reports that anthocyanin degradation is responsible for the colour change associated with the development of RDR (Salgado and Clark 2016; Pérez-Pérez *et al.*, 2018).

4.4.2. Physiochemical quality

The pH difference observed between affected and unaffected drupelets was relatively small, but statistically significant, with an average decline of 0.24 between FB and PR drupelets and 0.30 between FB and FR drupelets. The sensitivity of anthocyanin pigments to colour change at different pH levels through structural conformations of the anthocyanidin backbone is well documented (Brouillard 1983; Cabrita *et al.*, 2000; Castañeda-Ovando *et al.*, 2009). Though a drop in pH to below 3 has been reported tocause anthocyanins to shift to their red flavylium ion in isolated conditions of purified solutions (Cabrita *et al*., 2000), the co-pigmentation effect of various other polyphenols means that these pigments likely exist in equilibrium between their flavylium and quinonoidal base forms in blackberry cells *in vivo* (Brouillard, 1983; Davies and Mazza, 1993). The high concentration of these pigments in blackberries and their co-pigmentation with other polyphenols produces the desirable black colour of ripe blackberry fruit (Davies and Mazza, 1993; Mazza and Brouillard, 1990). Whilst the decline in pH observed in PR and FR may contribute to the observed colour change, on its own it is likely not significant enough of a shift to produce the drastic colour change seen during the development of RDR. The change in colour is more readily explained by the degradation of the pigments, which has been demonstrated in this report, and possibly accentuated by the decline in pH. The lower pH of FR and PR drupelets relative to FB drupelets may however contribute to the increase in electrolyte leakage and associated increase in membrane permeability, thereby influencing anthocyanin degradation. Decreased pH has been associated with increased pectin solubilisation in tomatoes (Chun and Huber, 1998), as well as increased plasma membrane permeability, anthocyanin leakage, and cell wall weakening in cherries (Winkler et al., 2015).

4.4.3. Electrolyte leakage

Electrolyte leakage was measured as a method of estimating cell plasma membrane damage to the fruit. The data showed significant increases in the EC of the solution from FB to PR and FR drupelets (Fig. 4-1). Electrical conductivity is a linear function of the concentration of ions within a solution, and using it to measure the electrolyte leakage from plant material is a commonly used method of indirectly assessing cell plasma membrane permeability (Bajji *et al.*, 2002; Concellón *et al.*, 2012). Generally, the measurement is carried out on discs of plant tissue of a predetermined thickness and size to reduce any variation caused by the surface area/size of the sample. This method was unsuitable for the current experiment due to the fluidic nature of blackberry flesh as well as the nature of RDR and the experimental aim of comparing whole FB to PR and FR drupelets. To minimise any variation using this method, drupelets selected for assessment were accurately weighed and selected so that all replicates of five drupelets were within 5 % of the same total mass, so drupelets with similar flesh to mass ratios were assessed. These data, in addition to the cell structural and chemical changes observed, indicated that tissue affected by RDR had been damaged, affecting the structural integrity and plasma membrane permeability. Membrane permeability can be influenced by a number of factors, many of which result from injury induced by stresses (Agarie *et al.*, 1998; Bajji *et al.*, 2002; Cox *et al.*, 1993). Previous studies have shown that electrolyte leakage increased with vibrational damage in pears (*Pyrus communis* L.) (Zhou *et al.*, 2007), increased postharvest damage to strawberries (*Fragaria vesca* L.) (Jiang *et al.*, 2001), and environmental conditions such as heat, drought, and chilling to other plant tissues (Agarie *et al.*, 1998; Bajji *et al.*, 2002; Campos *et al.*, 2003).

4.4.4. Firmness

The significant reduction in firmness of both PR and FR drupelets relative to FB drupelets (Fig. 4-2) demonstrated that RDR had a significant negative impact on physical quality in addition to the visual quality of blackberry fruit. Firmness is a key desirable trait for consumers (Basaran and Kepenek, 2011; Perkins-Veazie *et al.*, 1997), and soft fruit are more prone to leakiness and decay (Perkins-Veazie *et al.*, 1997). The impact of RDR incidence and severity on blackberry shelf-life has not been examined; however, the association with reduced firmness indicates that it may be a significant factor in reduced shelf life.

The cellular structural changes observed in this study were likely factors associated with the reduction in firmness of PR and FR drupelets. The effects of cell structure and plasma membrane permeability on fruit firmness in various horticultural products are well documented. As intercellular spaces increase and plasma membrane integrity decreases, fruit firmness is reduced in apples and pears (El Assi *et al.*, 1997; Khin *et al.*, 2007; Zhou *et al.*, 2011). Our results are consistent with this and suggest a drop in internal turgor pressure within affected drupelets. Our data may explain observations by Perkins-Veazie and Clark (2011) that drupelets affected by RDR contained less juice than unaffected drupelets. A potential mechanism behind this may be an increase in respiration in affected tissue causing moisture loss – a common occurrence after physical injury to fruits such as cherries (*Prunus avium* L.) and plums (*Prunus salicina* Lindl.) (Crisosto *et al.*, 1993; Martinez-Romero *et al.*, 2003; Wang, 1989). Perkins-Veazie *et al.* (1996a) also reported that reduced blackberry subjective firmness ratings were strongly correlated with ethylene production.

4.4.5 *Macro and microstructural observations*

The deformed and damaged cells observable in FR drupelets are further indicators of mechanical injury to the affected tissue (Figs. 4, 5). Physical rupture of cells and loss of cell-to-cell adhesion are likely responsible for the loss of firmness and degradation of anthocyanins in PR and FR drupelets. Intercellular spaces have been shown to increase susceptibility to bruise damage in apples and potatoes (*Solanum tuberosum* L.) through increasing the area for cell deformation (Bollen 2005; Garcia *et al.*, 1995; Opara 2007). Intercellular spaces present in the mesocarp of drupelets prior to damage may be a factor in individual drupelet susceptibility to RDR.

The undulations visible on the skin surface under ESEM are likely a result of a reduction in turgor pressure and are associated with the loss of skin firmness in affected drupelets. Similar physiological changes caused by exogenous sources of damage or stress have been reported as the mechanism behind fruit softening and altered mechanical properties in crops such as apples and strawberries (Chassagne-Berces *et al.*, 2009; Harker *et al.*, 2000; Marigheto *et al.*, 2004; Oey *et al.*, 2007).

Physical damage resulting in cellular decompartmentalisation of anthocyanins has been well established as a mechanism of colour change in a range of horticultural commodities, primarily through oxidative enzymatic actions or acid mediated hydrolysis separating the anthocyanin aglycone and sugar moieties (Clifford, 2000; Pifferi and Cultrera, 1974; Taranto *et al.*, 2017; Tomás-Barberán and Espín, 2001; Whitaker, 1995). The most commonly reported enzymes responsible for anthocyanin degradation are polyphenol oxidases and peroxidases, which can exist either latent or active in many plant tissues (Martinez and Whitaker, 1995; Whitaker, 1995). Whilst our data indicate an association between a loss of cell structural damage, anthocyanin degradation and RDR, further work is required to document the exact mechanism of anthocyanin degradation.

4.5. Conclusions

Blackberries affected by RDR undergo significant physiological and chemical changes resulting in a loss of fruit quality and marketability. The colour change observed is caused by a reduction in the concentration of total anthocyanins within affected tissue, with FR drupelets containing, on average, 42 % of the total anthocyanin concentration of FB drupelets. This colour change is accompanied by cell structural modifications including increased plasma membrane permeability, cell deformation, increased intercellular spaces and a minor pH shift. Within the anthocyanin profile, pelargonidin-3-glucoside and cyanidin-3-glucoside were the most readily degraded anthocyanins.

Hydroxylation of the aglycone, acylation of the sugar moiety, and larger sugar moieties significantly increased the stability of individual anthocyanins within the profile.

Our findings indicate mechanical injury causing cell decompartmentalisation and subsequent anthocyanin degradation as physiological processes occurring during the expression of RDR in blackberries. The data are from a single cultivar and further work is warranted to explore the extent of variation in the physiological mechanisms of the disorder among cultivars or breeding selections.

For breeders and producers, the selection of cultivars resistant to mechanical injury as well as developing management techniques to reduce damage to fruit during handling and transport may help reduce incidence and severity of the disorder in commercial settings.

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Chapter 5. Effects of climatic conditions during harvest and handling on the postharvest expression of red drupelet reversion in blackberries

After mechanical injury causing cell disruption was identified as a potential mechanism involved in RDR development in Chapter 4, this trial was designed to investigate the effects of injury inferred by handling fruit during harvest. Environmental conditions at harvest have been suggested by previous research, and anecdotally by producers, as contributing to high rates of the disorder, but no studies had investigated this thoroughly.

This chapter addresses the second goal of the project: to investigate physical and environmental factors influencing RDR incidence and expression. The fourth goal of the project is also addressed: offering techniques to reduce incidence of the disorder in commercial settings.

This chapter has been submitted for peer review as an original research paper.

Abstract

Red drupelet reversion (RDR) causes individual drupelets on blackberries to revert from black at harvest to a red colour postharvest, reducing the quality and marketability of the fruit. The objective of this trial was to assess the effects of time of harvest and associated climatic variables, as well as the handling of fruit during harvest, on postharvest RDR expression and fruit quality.

Fruit were harvested at the shiny black stage on 10 occasions over two days by one of two methods: either hand-harvested into shallow buckets and transferred to industry standard 125 g clamshell punnets (standard practice), or harvested carefully without handling by cutting the pedicel and placing each fruit into individual cotton-lined trays. After 24 h in cool storage, the number of partially red (PR) and fully red (FR) drupelets per fruit was counted, firmness was measured by compression, and skin firmness was measured by a penetrometer. Air and fruit skin temperature, relative humidity, vapour pressure deficit and soil water tension were all influenced by the time of day. A total of 85 % of fruit that were handled during harvest had at least one drupelet develop RDR, whilst only 6 % of fruit not handled during harvest had any RDR. In handled fruit, warmer skin temperature at harvest was associated with increased RDR incidence and severity ($P<0.001$). The skin firmness of fully black (FB) drupelets, measured by a penetrometer, also decreased significantly by an average of 0.56 N when harvested during warmer temperatures compared to fruit that was not handled. The data indicate that mechanical injury incurred during harvest is a major cause of RDR in fresh blackberries, and that harvest times associated with warmer temperatures result in significantly higher rates of RDR and reduced postharvest quality.

Keywords: Temperature, red drupelet, harvest time, bruising, firmness

5.1. Introduction

Blackberries (*Rubus* L subgenus *Rubus* Watson) are a popular summer fruit with an attractive dark colour and high antioxidant activity (Kaume et al., 2012; Wang and Lin, 2000). They are also one of the most perishable of commodities with shelf life often limited to less than a week in refrigerated storage due to rapid deterioration in quality (Joo et al., 2011; Perkins-Veazie et al., 1999). Red drupelet reversion (RDR), sometimes referred to as red drupelet disorder, red cell, or simply reversion, is a physiological occurrence which causes individual drupelets on a blackberry that are black at harvest to revert to a red colour, usually during cool storage postharvest. The disorder can reduce marketability of the fruit and lead to significant losses due to rejection or unsold product (USDA, 2018). Little research into RDR has been undertaken previously, however recent growth in worldwide production has seen the issue attract an increasing amount of attention from producers and researchers. The underlying mechanisms are uncertain, though it has been suggested that cell decompartmentalisation may play a role in the degradation of anthocyanins within affected drupelets (Salgado and Clark, 2016). Susceptibility to the disorder has recently been linked to cultivar firmness (Salgado and Clark, 2016), vibration injury during transport (Pérez-Pérez et al., 2018), and nitrogen fertiliser application rate (Edgley et al., 2016). Recent studies (Lawrence and Melgar, 2018; McCoy et al., 2016; Yin, 2017) have linked the time of harvest to rates of RDR expression with suggestions that temperature during harvest is a significant influence on the disorder, though this effect has been inconsistent and is cultivar specific. Identification of the factors involved in the development of RDR is of importance to the worldwide blackberry industry in order to develop standard management practices to reduce the incidence and severity of the disorder.

Several factors associated with the time of day can influence the physiology and postharvest quality of horticultural commodities. Environmental variables such as temperature, sun exposure, humidity, rainfall, and water availability have all been reported to affect firmness and bruise susceptibility across a range of horticultural commodities including strawberries, apples, and apricots (Hussein et al., 2018; Paull, 1999; Sams, 1999). Bruising of products is typically caused by one of three mechanistic sources: compression; vibration; or impact damage. Compression bruising occurs during harvest, handling, or by fruit pressing against one another during transport. Vibration bruising occurs through the rubbing of fruit against one another or the edge of surfaces, and impact bruising from sudden drops onto a surface or another fruit (Crisosto et al., 1993; Holt and Schoorl, 1982; Vergano et al., 1991). Pérez-Pérez et al., (2018) identified vibration damage to blackberries during transport as a factor associated with RDR development, however the susceptibility of blackberry fruit to damage from different mechanical sources or under different environmental conditions has not been thoroughly investigated. In strawberries, pulp temperatures above 30 ^{o}C have been reported to be positively correlated with compression damage but negatively correlated with impact damage (Ferreira et al., 2009). Compression damage has been reported to lead to 40 % more bruise volume in strawberries than impact damage of the same force (Holt and Schoorl, 1982). Compression damage is more likely to be caused during fruit harvest, whilst impact and vibration damage are more common during packing and transport (Aliasgarian et al., 2015; Crisosto et al., 1993; Garcia, Ruiz-Altisent and Barreiro, 1995). Aliasgarian et al., (2015) reported that 51 % of damage to strawberries including abrasions, bruises, and penetrations, were caused during harvest with 17 % occurring during packing and 32 % in delivery to market.

The optimisation of postharvest practices for fresh blackberries is of significant interest due to their high susceptibility to postharvest loss of quality and high market value. This study aims to investigate the association between harvest conditions and the incidence of postharvest RDR, and to assess the impact of hand harvesting on RDR development.

5.2. Materials and methods

5.2.1. Site and experimental design

Blackberries (*cv.* Ouachita) were harvested from a commercial berry farm located in Dunorlan, Tasmania, Australia (41.5 ° South, 146.6 ° East). Based on grower observations, the cultivar at this site had previously shown a medium-to-high susceptibility to RDR after commercial harvesting, and had a relatively small incidence of other pests and diseases. The site was flat with uniform soil and low wind exposure. Polyethylene tunnels on metal hoops covered the canes, with three east-west running rows per tunnel. The experimental site consisted of one tunnel within the site, with fruit sampled randomly at each harvest from each side of the three rows of canes.

5.2.2. Harvest

Fruit were harvested on 10 occasions during the 19th and 28th of February 2016. Day 1 (19th) had a below average daily maximum temperature (16.2 °C) and day 2 (28th) had an above average maximum temperature (26.0 °C). Harvests were carried out every two hours between 6 am and 2 pm. At each harvest time, 40 fruit per treatment were harvested from each side of three randomly selected 3 m sections of cane within the experimental plot.

Each row was considered to be a block for a randomised complete block design. All fruit harvested were at the 'shiny black' stage of development where fruit is fully black but with a visible glossy sheen (Perkins-Veazie et al.,, 1996),, which is the industry standard for commercial harvest. Selected fruit were otherwise free of pests, pathogens and insect damage.

Fruit were harvested using one of two methods:

1. Standard industry practice – berries were harvested by hand into shallow buckets and then transferred into 125 g clamshell punnets lined with a soaker pad.

2. Harvested without handling by carefully cutting the pedicle with Felco 100 Cut And Hold pruning shears (Felco, Australia) approximately 1 cm above the fruit receptacle, and placing each fruit into individual cotton wool-lined cells of 30 mm square seedling trays.

The skin temperature of every harvested fruit and 20 mid-canopy floricane leaves at each harvest time was measured using an infrared thermometer (HI 99556-10 Hanna Instruments, Australia). Fruit location within each punnet was marked and each fruit was given an individual identification number for matching temperature at harvest with postharvest quality. Following harvest, fruit was immediately placed into a 2 °C, 95 % relative humidity (RH) cooler and within 8 htransported on ice in coolers to the Tasmanian Institute of Agriculture in Hobart, Tasmania, Australia for storage and analysis.

5.2.3. Environmental variables

Air temperature and RH were monitored inside the polytunnel at mid-canopy level and outside in the shade of an adjacent windrow by three Hygrochron iButton (Maxim Integrated, USA) data loggers. Vapour pressure deficit (VPD) was calculated from logged data using the formula:

$$VPD = e_{sat} - e_{air}$$

Where e_{sat} is the saturation vapour pressure and e_{air} is the air vapour pressure (Measham, 2011; Murray, 1967). Soil moisture tension (kPa) was monitored prior to every harvest using soil tensiometers at soil depths of 15 and 30 cm.

5.2.4. Fruit quality analysis

All fruit quality testing was performed 24–30 h after harvest on fruit with a skin temperature of 4 ±1 °C. Three levels of reversion were used to assess the severity of individual drupelets expressing RDR. 'Fully red' (FR) drupelets were defined as having all of the visible flesh of the drupelet a distinct red colour. 'Partially red' (PR) drupelets were defined as having an amount of both red and black visible flesh on the drupelet. Fully black (FB) drupelets unaffected by RDR were not counted per fruit but made up the remainder of the drupelets on each fruit. The red drupelet index (RDI) score for each fruit was calculated using the formula:

$$RDI\ =\ number\ of\ PR\ drupelets + (2 \times number\ of\ FR\ drupelets)$$

Skin firmness was measured on two FB drupelets on each fruit using a GUUS texture analyser with a 3 mm probe set to descend at 25 mm s^{-1} with a minimum force threshold of 0.294 N, and the average of the two readings per fruit was taken. Whole fruit firmness was measured by compression (2 mm) using a Firmtech II firmness tester (Bioworks Inc, Wamego, KS, USA) and expressed as Newtons (N) of force used.

5.2.5. Statistical analysis

R version 3.3.1 (R Core Team 2017) was used for all statistical methods. RDI data were examined using a generalised linear mixed model (GLMM) approach since data were non-normally distributed. Likelihood ratio tests and Akaike information criterion were used to choose the best fitting model between zero-inflated negative binomial, hurdle negative binomial, and quasi-poisson distributions. The best fitting, zero-inflated negative binomial model was fitted using the 'glmmTMB' package (Brooks *et al.*, 2017). The effects of harvest treatment and environmental variables on other postharvest quality parameters were examined using analysis of variance (ANOVA) techniques with Tukey Honest Significant Difference (HSD) post-hoc tests. A significance level of $P < 0.05$ was used for all analyses.

5.6. Results and discussion

5.6.1. Diurnal variation of environmental conditions

The average temperatures of the air, the floricane leaves, and the fruit skin were not significantly different between the 6 am and 8 am harvest times on either day, but as the air temperature increased during later harvests these values became significantly different (Fig. 5-1). The differential between the skin and air temperatures also increased in significance ($P < 0.01$) on both days between the 10 am, 12 pm, and 2 pm harvests, indicating that fruit and leaf temperatures rose at the highest rate of these variables. The higher fruit and leaf temperatures compared to the tunnel air temperature was likely due to solar activity warming the fruit directly, an effect which has been previously reported for spherical fruit (Smart and Sinclair, 1976; Woolf and Ferguson, 2000).

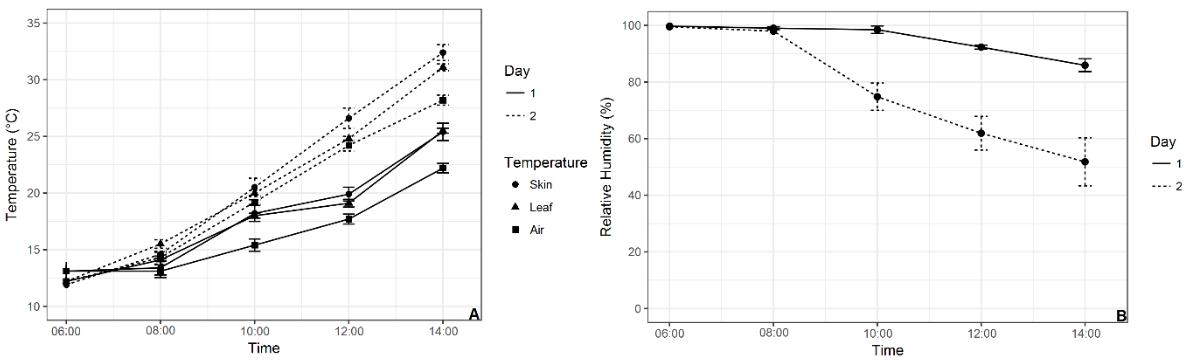
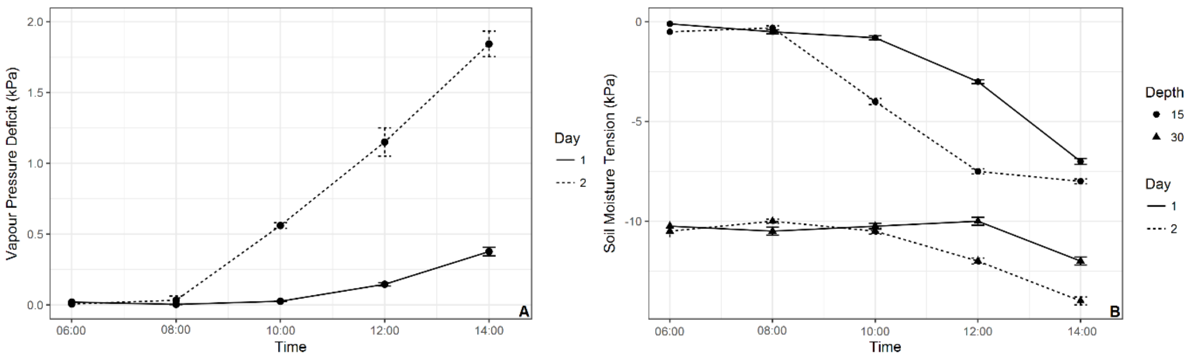


Fig. 5-1. Diurnal variation at each harvest time of: temperatures (^{o}C) outside, inside the poly tunnel, of fruit skin, and of floricane leaves (A) and; RH (%) at each harvest (B).

Relative humidity inside the polytunnel was at 100 % overnight and remained above 97 % until 8 am on both days when the air temperature was below 15 °C. On day 1, the RH declined linearly at -3.15 % h^{-1} after 10 am to 85.9 % at 2 pm. On day 2, RH declined between 8 am and 10 am to 74.8 %, and then to 61.9 % and 51.8 % at 12 and 2 pm (Fig. 5-1). The changes in temperature and RH resulted in significant differences in the VPD inside the polytunnel between afternoon and morning harvests on each day, as well as between days (Fig. 2). The VPD on day 1 increased between 10 am and 2 pm from 0.03 kPa to 0.38 kPa but remained below 0.5 kPa for all harvests.

The VPD on day 2 was significantly higher than day 1 after 10 am, increasing to 0.56 kPa at 12 pm,



1.15 kPa at 2 pm, and 1.84 kPa at 4 pm (Fig. 5-2).

Fig. 5-2. Vapour pressure deficit (kPa) inside the polytunnel (A); and soil moisture tension (kPa) at 15 and 30 cm soil depth (B) at each harvest time.

Changes in soil moisture tension at 15 and 30 cm soil depth on both days followed a similar trend to temperature and VPD. No significant differences were observed at either depth before 10 am, but at later harvests soil water became less available at 2 pm on day 1, and after 12 pm on day 2 (Fig. 5-2). The fluctuations in soil water at both 15 and 30 cm indicate a reasonably responsive soil to the diurnal temperature fluctuations and plant water use.

All the measured environmental factors followed similar trends over both days, which is to be expected with such intrinsically linked variables. Little information exists regarding the effects and mechanisms of these variables during harvest on postharvest quality and bruise susceptibility of *Rubus* fruit, but previous reports have established the potential for these factors to influence the postharvest quality of other horticultural commodities (Ferreira et al., 2009; Prange and DeEll, 1995).

5.6.2. Effects of environmental conditions on RDR

Harvest technique was the most significant factor associated with the presence or absence of RDR on a fruit as shown by the RDI values (Table 5-1). Skin temperature of handled fruit significantly increased the severity of RDR as temperature rose (Fig. 5-3). The inclusion of other environmental variables in the model beyond skin temperature did not improve the model fit.

Table 5-1. Coefficient estimates and significance of the best fitting model.

Count model coefficients	Estimate	Std. Error	Z Value	Pr(>|z|)
Harvest treatment	-3.3	0.45	-5.97	<0.001
Skin temperature	0.06	0.005	10.71	<0.001
Zero inflation coefficients				
Harvest treatment	3.34	0.68	4.90	<0.001

[1]Model: $RDI = Harvest\ Treatment + Skin\ Temperature + Firmness + Random\ Effect(fruit)$
[2]Random Effect (Fruit)
[2]Log-likelihood: -1168 on 9 df

Soil tension at 30 cm was nearly significant (P = 0.10); however, model fit was not improved with the inclusion of this variable. Given that soil moisture and VPD remained relatively unchanged at seven out of the 10 harvest times, more data points may have resulted in identifying the significance of these variables on RDR.

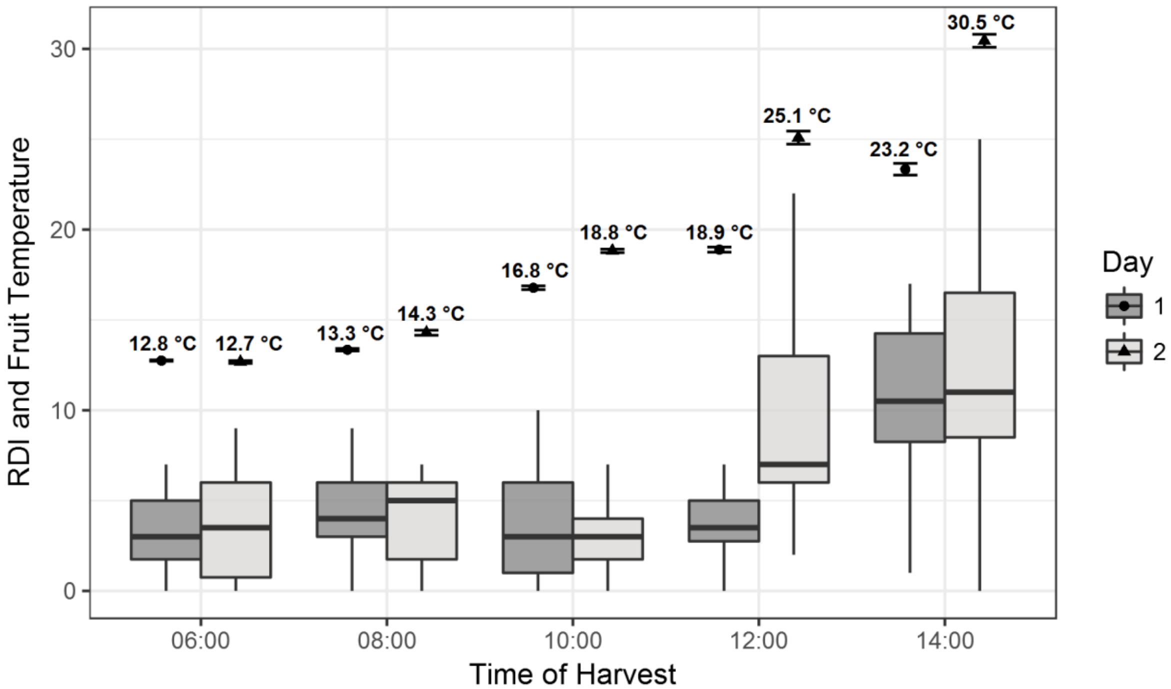


Fig. 5-3. Red drupelet index of fruit from harvest treatment 1 and the mean fruit skin temperature (°C) at each harvest. Max/min values, medians, and quartiles are shown for RDI; mean ± standard deviation are shown for temperatures. n = 40 per harvest.

These results are consistent with those reported by both McCoy *et al.* (2016) and Yin (2017) for the effects of harvest time and temperature on RDR expression. McCoy *et al.* (2016) reported that fruit from eight cultivars that was harvested at 7 am with a mean skin temperature of 23.1 °C had significantly lower incidence of RDR than fruit harvested at 10 am, 1 pm, and 4 pm – all of which had skin temperatures of above 29 °C. Yin (2017) reported that a harvest time of 7 am produced significantly cooler fruit than harvests at 12 pm and 4 pm, and that most cultivars showed a significant increase in the percentage of red drupelet expression with later harvest times. In these two trials the largest increase in fruit temperature occurred between the morning and midday harvests and corresponded with a significant increase in RDR. Lawrence and Melgar (2018) found that the effects of harvest date, harvest time and a delay to storage varied significantly among 10 blackberry cultivars, but that a 7 am harvest time did not affect RDR in 'Ouachita' fruit compared to 10 am, which is inconsistent with our results. The authors did suggest confounding effects of RH, storage time and plant water status, suggesting the need for further research into these variables. Further to this, blackberry canes in our study were grown under polythene tunnels as opposed to open field in these comparison studies which may further compound the effects of various environmental factors on reversion incidence.

Whilst the effects of soil moisture status and VPD have not been investigated thoroughly, there is some consistency across climates, cultivars and seasons indicating a significant influence of fruit temperature on postharvest RDR incidence. In our results, these conditions were also associated with higher soil moisture tension; however, more data are needed from a wider range of environmental conditions to investigate and separate any confounding effects of these variables. The effects of soil moisture on overall postharvest quality in blackberries is not currently well understood (Strik and Finn, 2011), though increased soil moisture during harvest has previously been associated with decreased firmness in blueberries (Ehret *et al.*, 2012) and strawberries (Sistrunk and Morris, 1985). As soilless production of blackberries becomes more common (Carlen and Crespo, 2012), the importance of understanding how water availability and plant water status may impact postharvest performance will increase due to the susceptibility of these systems to rapid fluctuations in water availability.

5.6.3. Effects of harvest treatment on RDR

Fruit from harvest treatment 1 had relatively high total incidence of RDR, with an average of 85 % of fruit across both days and all harvest times having at least one PR drupelet 24 h after harvest (Table 5-2). Mean RDI was not significantly different between days at 5.32 on day 1 and 6.40 on day 2. These rates of incidence for harvest treatment 1 are consistent with those seen previously for commercial 'Ouachita' blackberries grown in the Tasmanian climate (Chapter 6).

Table 5-2. Percentages of fruit with at least one reverted drupelet per harvest treatment over both harvest days. n = 200 fruit per harvest treatment and day. Data are averaged from all harvests on each day.

Day	Harvest treatment	Fruit with 1+ red drupelets (%)	Fruit with 3+ red drupelets (%)	RDI
1	1	84.0	72.0	5.32 ± 0.52 a
1	2	4.1	0	0.041± 0.02 b
2	1	86.0	81.0	6.40 ± 0.57 a
2	2	7.9	0	0.083 ± 0.04 c

Means followed by different letters in RDI column were significantly different at P<0.05.

Fruit harvested using treatment 2, with careful precautions against physically damaging the fruit during harvest and transport, showed a dramatic reduction in postharvest expression of RDR in all fruit assessed. This harvest treatment produced fruit with an average of only 6 % of fruit showing any RDR (Table 5-2) and no fruit showing 3+ reverted drupelets. Day 1 had significantly fewer fruit with reverted drupelets from this treatment than day 2 (4.1 % vs 7.9 %) and higher RDI (0.04 vs 0.08), though still produced no fruit with 3+ reverted drupelets. No FR drupelets were observed on fruit from this harvest treatment. Though this is an impractical method of harvest for any commercial scenario, the results highlight the impact that harvest and transport conditions can have on postharvest fruit quality.

5.6.4. Effects of environmental conditions and harvest treatment on firmness

No significant differences in skin firmness were observed between harvest treatments when fruit temperature was below 23 °C (day 1, 6–12 pm, day 2, 6–10 am). However, when temperature and soil moisture tension increased there were significant reductions (P<0.01) in skin firmness (Fig. 5-4). In associated research we demonstrated that drupelets affected by RDR also required less force for skin penetration than healthy drupelets (Chapter 4). Penetrometer tests in this trial were carried out on drupelets unaffected by RDR, so this reduction indicates that damage is incurred by handling during harvest that reduces skin firmness, but does not result in RDR expression. Fruit firmness has previously been linked to postharvest shelf-life of small fruits as well as overall fruit quality as perceived by customers (Cao *et al.*, 2010; Ehlenfeldt and Martin, 2002; Hernández-Muñoz *et al.*, 2008), thus this result may have further implications for quality than RDR expression alone.

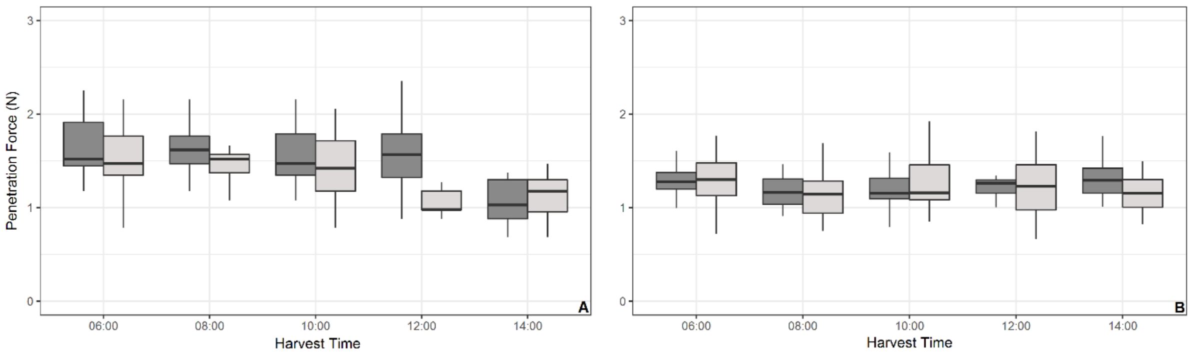


Fig. 5-4. Skin firmness (N) of FB drupelets from harvest treatment 1 (A) and 2 (B). Max/min values, medians, and quartiles are shown.

Compression firmness was not significantly different between harvest times or treatments with a large amount of variation between readings for all fruit. Mean compression values (N) were 1.22 ± 0.27 from harvest treatment 1 and 1.35 ± 0.22 from harvest treatment 2 across all harvests (data not shown). Whilst the Firmtech II device has been used successfully in previous studies involving blackberries to rapidly obtain firmness evaluations between breeding lines (Perkins-Veazie, *et al.*,, 2000), our results suggest that data obtained from the penetrometer device may be better suited for differentiating fruit quality in this scenario. Both Salgado and Clark (2016) and McCoy *et al.* (2016) reported that a compression device gave more reliable results for blackberries; however, the make and models of equipment used (iCon Texture Analyzer, Texture Technologies Corp. Hamilton, Massachusetts, USA) differed to the current trial.

Many physiological and environmental variables can affect fruit firmness (Sams 1999), which may increase the difficulty of obtaining precise and comparable results between harvests and production climates for these variables. For the instruments used in this study, a penetrometer device with a flat 3 mm probe appeared to produce the most precise data.

There were no changes to skin firmness with the time of day in harvest treatment 2, and these values were also not significantly different to fruit harvested at <23 °C from treatment 1. This indicates that variables associated with harvest time did not affect postharvest fruit firmness alone, but that the reduction in skin firmness observed in fruit from harvest treatment 1 was possibly associated with damage caused during harvest and transport. As firmness and skin texture are factors in fruit quality (Perkins-Veazie *et al*., 1996) these findings suggest that harvest time may be important for broader fruit quality and shelf-life as well as RDR development.

The results for harvest time and postharvest quality indicate that manipulation of harvest practices may be an option for producers to reduce the impact of mechanical injury during harvest. Harvesting in the early morning before field temperatures rise is an obvious method to potentially reduce the incidence of RDR. The data from the current trial as well as previous work (McCoy *et al.*, 2016; Yin, 2017) indicate that fruit harvested earlier in the day consistently had lower rates of the disorder and that this was associated with lower fruit temperatures at harvest. Reducing the temperature of fruit in the field through utilising shade from cloth or cane management techniques may provide options to reduce incidence. This technique may need to be approached with caution, however, as light intensity affected by shading is a significant factor in nitrogen uptake, anthocyanin development, and carbohydrate supply that drives yield in a range of other crops such as grapes (Keller *et al*.,1998; Keller and Hrazdina, 1998), and strawberries (Demirsoy *et al.*, 2007).

Punnet design should also be considered as a possible management factor to reduce RDR incidence. The commonly used 125 g square clamshell punnets typically contain two layers of fruit resting on top of one another resulting in a source of compression and vibrational damage during transport.

As well as this, as the emergence of larger-fruiting cultivars may have led to more fruit touching the punnet lid and sides, and heavier fruit causing more compression damage. A change to this commonly used punnet design may reduce the amount of vibrational and compression damage caused during packing, transport and handling. This issue has been discussed in recent years within the strawberry industry, with punnet design and padding being identified as key factors in reducing losses, caused in part by mechanical damage (Chaiwong and Bishop, 2015; Mirzaee and Bishop, 2009). Ideally, producers should use punnets which minimise fruit-on-fruit and fruit-on-punnet contact, whilst still maintaining adequate airflow and padding to preserve fruit quality. Additional factors to reduce injury, such as the type of padding or other design modifications, present an opportunity for further research to be undertaken to optimise packaging for blackberries.

5.7. Conclusions

Fruit and leaf temperatures, VPD, and soil moisture availability varied with the time of day that blackberries were harvested. Fruit handling at harvest was identified as a significant factor in the development of RDR; 85 % of fruit that was handled developed RDR, whilst only 6 % of fruit that was not handled developing any incidence of the disorder.I Increased fruit skin temperature during handling significantly increased the severity of the disorder in affected fruit. This effect was most notable at skin temperatures exceeding 23 °C, which is consistent with previous reports for flesh temperatures associated with increased rates of RDR across cultivars, seasons, and environments (McCoy *et al.*, 2016; Yin 2017). This consistency in results with previous studies suggests that the environmental conditions associated with later in the day and warmer harvest times are major factors in increased rates of postharvest RDR. To reduce incidence and severity of the disorder, fruit should be handled minimally during harvest, and harvested early in the day during cool conditions. Handling during harvest was also associated with softer skin in drupelets not affected by RDR.

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Chapter 6. Nitrogen application rate and harvest date affect red drupelet reversion and postharvest quality in ‘Ouachita’ blackberries

This chapter addresses the second aim of the project: to investigate the effects of nitrogen application rates on RDR incidence. Supplementary data for this chapter is contained in Appendix D.

This chapter has been submitted for peer review as an original research paper.

Abstract

Red drupelet reversion (RDR) is a postharvest physiological disorder in blackberries that causes fruit that is black at harvest to subsequently turn red. This trial aimed to investigate the effects of nitrogen (N) fertiliser application rate on the expression of RDR and postharvest fruit quality. Nitrogen was applied weekly during the growing period via fertigation at a low, medium, and high rates (53, 106, and 212 kg N ha^{-1} respectively) to ‘Ouachita’ blackberries in 2016 and 2017. Yield, RDR, and postharvest quality were assessed. Harvest date, N application rate, and fruit mass were significant factors in the postharvest expression of RDR. In both years, fruit from the high N treatment exhibited significantly increased incidence and severity of RDR relative to the other two N application rates. Fruit temperatures during harvest of more than 23 °C were associated with higher incidence and severity of RDR in 2017, and smaller fruit were more likely to have no RDR in both years. The high N treatment produced more fruit than the low N treatment in 2016, and more and heavier fruit than both other treatments in 2017.

Keywords: Fertigation; temperature; fruit mass; mechanical injury

6.1. Introduction

Plant nutrition is an important factor in horticultural production, with nutritional status and management techniques having the potential to significantly influence many aspects of postharvest fruit quality. Nitrogen (N) is one of the most abundant plant nutrients and is required in high concentration for plant growth and development. An adequate supply is needed for bud and fruit development, yield, fruit quality and resistance to disease (Dordas, 2009; Marcelle, 1995; Prange and DeEll, 1995).

Red drupelet reversion (RDR) in blackberries (*Rubus* L. subgenus *Rubus* Watson) is a physiological disorder causing individual or groups of drupelets on a fruit that are black at harvest to revert to red, usually within 24 h of postharvest cool storage. The influence of preharvest plant nutrition on the incidence and severity of RDR has not previously been reported. However, it has been speculated that an oversupply of N may play a role in the expression of the disorder. In a preliminary report, we suggested that N application rates may play a role in RDR incidence, but also emphasised the need for further research into the relationship between these factors and the associated mechanisms (Appendix D.2).

It has recently been established that mechanical injury during harvest and transport is associated with RDR development (Chapters 4, 5; Pérez-Pérez *et al.*, 2018), and that harvest conditions associated with warm fruit temperatures increase the incidence and severity of RDR (Chapter 5; McCoy *et al.*, 2016; Yin, 2017). In related work, we demonstrated that the degradation of anthocyanin pigments is the cause of the colour change associated with RDR, with affected drupelets containing on average 43 % of the total anthocyanins present in black drupelets (Chapter 4). Salgado and Clark (2016) reported that blackberry flesh texture and firmness may influence cultivar susceptibility to RDR. Fruit cultivars with 'crispy', firm skin had fewer intercellular spaces, were firmer and exhibited significantly lower incidence of the disorder.

Nitrogen fertiliser application has been linked to susceptibility to mechanical damage in a range of fruit crops (Hussein *et al.*, 2018; Prange and DeEll, 1995; Sams, 1999). Generally, it is thought that N oversupply can stimulate excessive vegetative growth, which can in turn negatively affect fruit development leading to softer fruit (Hussein *et al.*, 2018; Mengel *et al.*, 2001). A relationship between N oversupply and the reduced physical quality of fruit has been demonstrated in a number of horticultural crops, including apples (Nava *et al.*, 2007; Neilsen *et al.*, 2009), cherries (Swarts *et al.*, 2017), and strawberries (Lanauskas *et al.*, 2006; Miner *et al.*, 1997; Nestby *et al.*, 2005; Shoemaker and Greve, 1930).

Nitrogen application rates and the source of fertilisers have been implicated in other aspects of blackberry postharvest quality. Ali (2012) reported that increasing N supply from 60 kg ha^{-1} to 100 kg ha^{-1} led to increased fruit sugar content, increased fruit pH, and when combined with high rates of potassium (K) supply, increased fruit antioxidant content. Moreover, Alleyne and Clark (1997) observed that increasing N application rates from 0 kg ha^{-1} to 56 and 112 kg ha^{-1} did not affect sugars or titratable acidity (TA), but did significantly increase pH in 'Arapaho' blackberries.

Previous reports regarding the effects of N fertilisers on physical quality and yield in *Rubus* crops have been inconsistent (Strik, 2008). Nelson and Martin (1986) reported that N application rate had no consistent effect on fruit firmness in 'Thornless Evergreen' blackberries, but an N rate of 67 kg ha^{-1} produced the highest total yield in 'Arapaho' blackberries. Naraguma and Clark (1998a) reported no significant differences in terms of yield, berry mass, or primocane number between 0, 56, and 112 kg N ha^{-1} applications in 'Arapaho' blackberries. Strik (2008) reviewed the inconsistencies in the literature and suggested that variability in soil fertility, N rates used, cultivar and length of trials may be contributing factors. Although the literature to date regarding the effects of N application rates on yield and quality is inconclusive, it is evident that the source and rate of N fertiliser can significantly influence aspects of postharvest blackberry quality. Additionally, while no reports of N supply being linked to RDR incidence currently exist in the literature, we hypothesise that N application rates could impact susceptibility to RDR induced by mechanical injury by affecting fruit firmness or textural properties. Accordingly, this trial aimed to investigate the effects of N application rates on postharvest blackberry quality with a focus on the development of RDR.

6.2. Materials and Methods

6.2.1. Experimental Design

The experimental layout for the trial consisted of a randomised complete block design, with each of three polytunnels treated as a block. Each block contained three 106 m long rows of 'Ouachita' blackberry canes spaced at 2.5 m intervals. Each row was treated with a randomly allocated treatment of low (53 kg ha^{-1}), medium (106 kg ha^{-1}), or high (212 kg ha^{-1}) N fertiliser rate each season over a two-year period in 2016 and 2017. The treatments were applied weekly as liquid calcium nitrate via drip fertigation from November through March. All other agronomic factors including other nutrient fertiliser regimes, irrigation, pruning, pest, and disease control were managed as per industry standard for tunnel-grown 'Ouachita' blackberries (C Folder, personal communication, November 2016). Temperature (°C) and relative humidity (%) throughout the season was logged hourly in each tunnel by iButton DS1923 Hygrochron data loggers (Maxim Integrated, USA), which were hung from the middle cable of the trellis system. Two loggers were placed outside the tunnels in the shade of adjacent windrows to record ambient outside temperatures.

Fruit harvest

Fruit was harvested every 15 days for the length of the commercial harvest period. The 2016 harvest commenced on January 11th through to March 12th for five harvests, and the 2017 season commenced on 15th January through to March 30th for six total harvests. At each harvest, every fruit at the shiny black or dull black maturity stages was hand-picked from each side of three randomly selected 4 m sections of cane per treatment row, not including a 5 m buffer zone at the end of each row. Fruit was harvested between 8-11 am at each date and was transported within 3 h in a cooler with ice to the Tasmanian Institute of Agriculture in Hobart for storage at 4 °C and 95 % relative humidity until analysis. The skin temperature of 10 random fruit at harvest was measured using a HI99556 infrared thermometer (Hanna Instruments, Keysborough, VIC, Australia).

6.2.2. Physical quality

Subsamples of 20 fruit at the shiny black stage of maturity were taken from each replicate to evaluate for physical quality characteristics after 24 h in storage (N = 60 per treatment per harvest), and a further 40 fruit were frozen at -24 °C for later physiochemical quality analysis. Fruit for physical analysis were weighed on Mettler Toledo Scientific Balance scales (Mettler Toledo, Columbus, Ohio, USA), with average mass and total fruit count from harvested sections used to approximate yield (g m^{-1} of cane). Fruit firmness was measured using a Firmtech II firmness tester (Bioworks Inc, Wamego, KS, USA) and expressed as the force (N) required to compress fruit 2 mm.

For RDR, the number of partially red (PR) and fully red (FR) drupelets on each fruit was counted. Drupelets were considered FR only if 100 % of the visible tissue was a red colour, and PR if any amount of flesh less than 100 % of the visible tissue had changed colour. The number of PR and FR drupelets on each fruit was used to calculate a single red drupelet index (RDI) score per fruit using the following formula that scored FR drupelets at twice the severity of PR drupelets:

$$RDI \ = \ number\ of\ PR\ drupelets + (2 \times number\ of\ FR\ drupelets)$$

6.2.3. Physiochemical quality

Frozen samples were brought to 4 °C in a refrigerator overnight and then homogenised using a Retsch Grindomix GM 200 knife mill (Retsch, Waan, Germany) for 30 s. The homogenate was centrifuged for 10 min at 4000 rpm at 4 °C to obtain a clarified juice sample. Total soluble solids (TSS) was measured as ^{0}Brix using a Shibuya Optical hand-held refractometer (Shibuya Optical Co., Ltd., Wako-Shi, Saitama, Japan). Titratable acidity (TA) and pH were measured using a Metrohm 702 SM Titrino automated titrator (Metrohm, Gladesville, NSW, Australia), with TA being expressed as percentage of citric acid equivalent.

A further subsample of fruit from the first, third, and final harvest of each season was transported immediately following harvest to AgVita Analytical (Devonport, Tasmania, Australia) for macronutrient concentration analysis (N, P, K, Ca) by dry ash analysis. Primocane leaves including petioles were also sampled from mid-canopy height two weeks after the final harvest (March 12th 2016, March 30th 2017)of both years for macronutrient analysis.

6.2.4. Statistical analysis

RDI data were analysed using a generalised linear mixed models (GLMM) approach in R version 3.5.1 (R Core Team 2018). A zero-inflated negative binomial model was chosen by comparing different models using likelihood ratio tests for nested models and differences in the Akaike information criterion for non-nested models. The zero-inflated negative binomial model was fitted with the glmmTMB package (Brooks *et al.*, 2017). Model suitability was evaluated using the DHARMa package (Hartig 2017) by testing residuals from 1000 simulations against observations from the real data set and using the Kolmogorov-Smirnov (KS) test. Effects of N treatment and pick date on fruit quality measurements from subsamples were tested by two-way analysis of variance (ANOVA) with Tukey's Honest Significant Difference (HSD) post-hoc tests. A significance level of $P<0.05$ was used for all analyses.

6.3. Results

6.3.1. Red Drupelet Reversion

The best fitting, zero-inflated negative binomial regression model (KS test statistic = 0.73), indicated significant effects of harvest date ($P<0.01$), berry mass ($P<0.01$), and N treatment ($P<0.05$) on RDI, whilst time of year and berry firmness did not have a consistent effect on RDI (Supplementary Table D-1). Harvests three, four, and five in 2016 and one, three, and four in 2017 had significantly more fruit with zero RDI, which reduced the overall RDI at these harvests ($P<0.05$) (Fig. 6-1). Smaller fruit (<6.3 g) had a higher likelihood of containing no reverted drupelets ($P<0.05$). The high N treatment had elevated RDI relative to both other N application rates at the first four harvests in 2016 and the final three harvests in 2017 (Fig. 6-1). There were no significant differences in RDI between the low and the medium application rate treatments at any harvest.

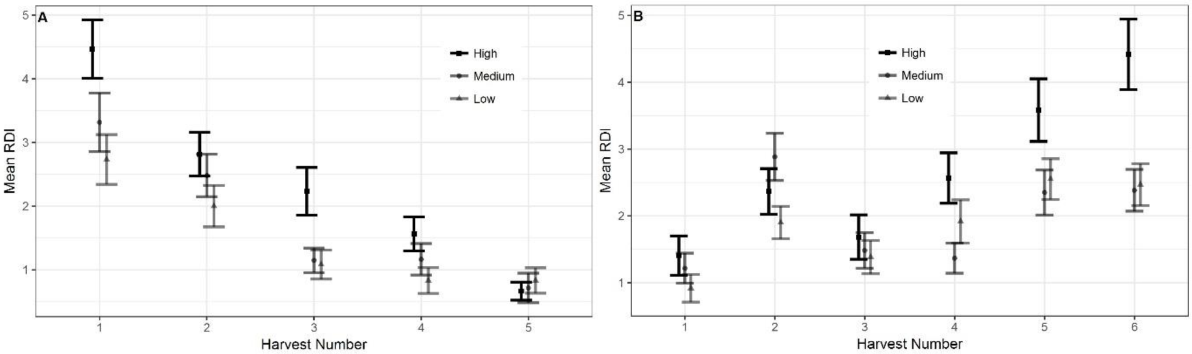

Fig. 6-1. Mean RDI at each harvest in season 2016 (A) and 2017 (B). Means ± standard deviations are shown.

In the 2016 season, RDI of fruit from all treatments was highest at harvest one and declined significantly between harvests one and five (P<0.01), with very low incidence of RDR in fruit from harvest five (Fig. 1). In 2017 this trend was not observed: RDI was lowest at the beginning of the season and was higher at later harvests (Fig. 1). In 2016, 57.2 % of the total fruit harvested for the season across all treatments had some incidence of RDR; and in 2017, 63.2 % of fruit across all treatments had some incidence (data not shown).

At all harvests, outside and inside tunnel ambient air temperatures were closely related to fruit skin temperature, with the mean skin temperature generally reflecting the inside air temperature in cooler conditions, but exceeding it as the ambient air temperature increased (Table 6-1).

Table 6-1. Outside ambient air, inside tunnel air, and mean fruit skin temperatures (°C) at each harvest in both seasons

2016 Harvests						
Location	**1**	**2**	**3**	**4**	**5**	
Outside tunnel	13.9	19.7	19.8	20.8	15.8	
Inside tunnel	15.6	22.1	23.2	21.2	16.7	
Berry skin	15.2	23.1	25.0	22.8	17.0	
2017 Harvests						
Location	**1**	**2**	**3**	**4**	**5**	**6**
Outside tunnel	16.8	19.2	17.4	18.7	21.0	21.0
Inside tunnel	22.2	23.2	20.7	18.7	23.2	23.7
Berry skin	22.6	27.4	22.5	18.1	27.0	27.1

6.3.2. Fruit size and total yield

In 2016, harvests one and two contributed the most to the cumulative yield (Fig. 2), whilst the final three harvests produced significantly less fruit (P<0.01). There were no yield differences between N treatments at any individual harvest, but cumulative yield was significantly increased (P<0.05) between the low (2127 g m^{-1} cane) and high (2466 g m^{-1}) treatments for the 2016 season. The 2017 total cumulative yield for all harvests from the high treatment (4644 g m^{-1}) was significantly greater than both the low (3259 g m^{-1}) and medium (3475 g m^{-1}) treatments (P<0.01). A significant difference in yield between N treatments at individual harvests was also observed in 2017, where the high treatment produced greater yields than both other treatments at harvests three through six (P<0.05). The medium and low treatments were not significantly different at any individual harvest or in cumulative yield in 2017.

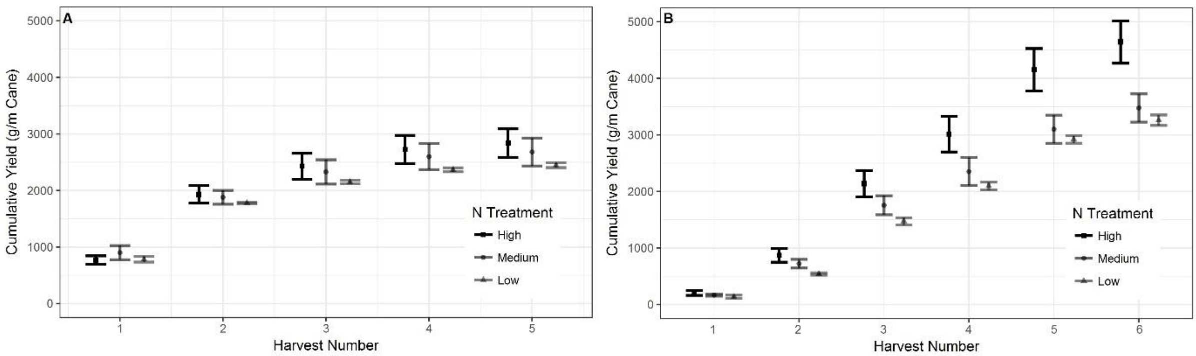

Fig. 6-2. Average calculated cumulative yield (g m^{-1} cane) at each harvest for the 2016 (A) and 2017 (B) seasons. Cumulative means ± standard deviations are shown.

In 2016 fruit mass from all treatments was highest at the beginning of the season and decreased gradually (Fig. 3), representing a significant difference between harvests one and five (P<0.01) for all treatments. No differences in fruit mass between treatments were observed in 2016. In 2017, average mass across treatments increased between harvests one and three (P<0.05) then declined significantly between harvest three and harvest six (P<0.05). The high treatment produced significantly larger fruit (P<0.05) than the low treatment in 2017 at harvests two, three and four, and the average mass over the whole season was also higher. No other interactions between the N application rate and mass were observed in either year.

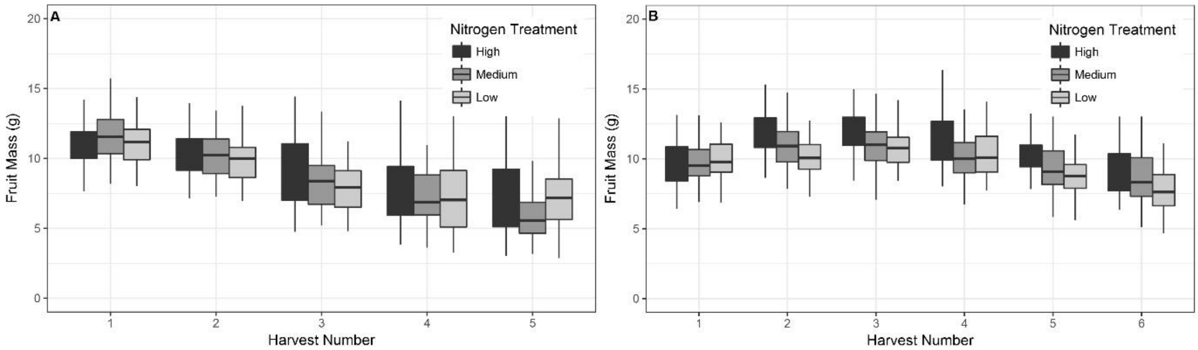

Fig. 6-3. Mean fruit mass (g) at each harvest during the 2016 (A) and 2017 (B) seasons. Min/max values, medians, and quartiles are shown.

6.3.3. Firmness

No significant relationship was identified between firmness and N application rate at any harvest date in either year (Fig. 4). There was a trend for softer fruit at the end of the season compared to the beginning in both years (P<0.05), and values between the two seasons were similar at the corresponding harvest periods.

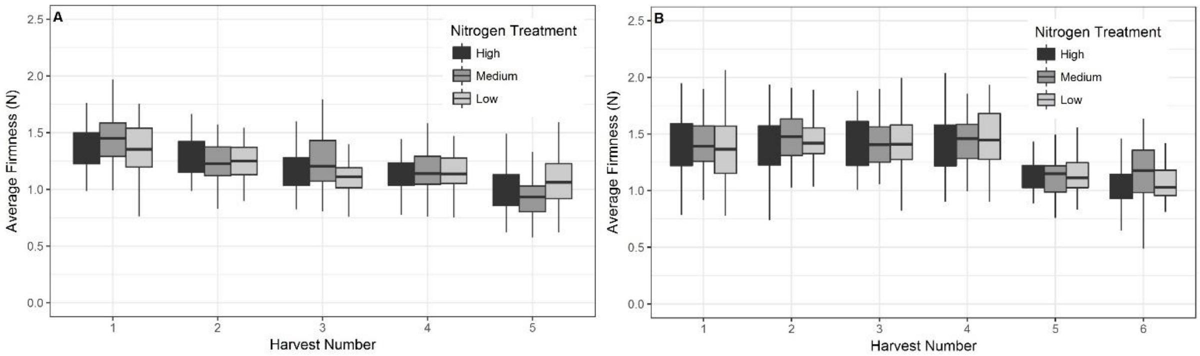

Fig. 6-4. Force (N) required to compress fruit 2 mm 24 h after harvest at each harvest date in 2016 (A) and 2017 (B). Min/max values, medians, and quartiles are shown.

6.3.4. Physiochemical properties

Over both seasons the only significant difference between N application rates for any of the physiochemical variables measured was a reduced pH (P<0.05) in fruit from the low treatment at harvest one (2.92) in 2016, and harvests one (2.86) and three (3.07) in 2017 (Fig. 6-5).

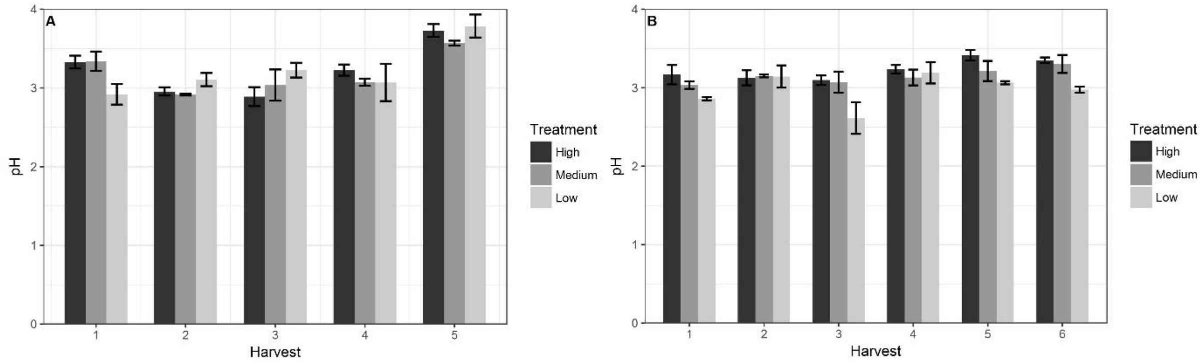

Fig. 6-5. Mean pH at each harvest in 2016 (A) and 2017 (B). Means ± standard deviations are shown.

During the 2016 season the TSS concentration from all N treatments dipped at harvest four, while the TSS:TA ratio was significantly lower in the middle of the season and higher during the first and fifth harvests ($P<0.05$) (Supplementary Data, Table D-2). In 2017, the TSS and TSS:TA ratio also declined between the beginning and end of the season ($P<0.05$). Monomeric anthocyanin concentration in 2016 increased between the beginning and end of the season, with an average increase of 26 % between harvests one and five across all N treatments (Supplementary Data, Table D-2). In 2017, monomeric anthocyanin concentration was highest at the beginning and end of the season, with a dip in the mid-season harvests three and four (Supplementary Data, Table D-3). In both seasons, anthocyanin concentration was inversely correlated with fruit size ($P<0.05$).

6.3.5. Fruit and plant N concentrations

Increased N application rate significantly increased fruit N concentration in the early and late season fruit in 2016, as well as in the early season fruit in 2017 (Table 6-2). No consistent trends were observed for P, K or Ca concentrations in fruit between N treatments or harvest year (Supplementary Data, Table D-4).

Table 6-2 Mean N concentration (%) in fruit over the course of each season and primocanes post-season

2016 Harvests				
N Treatment	**1**	**3**	**5**	**Post-season Primocane**
212 kg N ha^{-1}	1.17 ^{a}	1.15 ^{a}	1.20 ^{a}	2.26 ^{a}
106 kg N ha^{-1}	1.05 ^{b}	1.14 ^{a}	1.10 ^{ab}	1.98 ^{a}
53 kg N ha^{-1}	1.01 ^{b}	1.16 ^{a}	1.05 ^{b}	2.27 ^{a}
2017 Harvests				
N Treatment	**1**	**3**	**5**	**Post-season Primocane**
212 kg N ha^{-1}	1.31 ^{a}	1.22 ^{a}	1.08 ^{a}	2.51 ^{a}
106 kg N ha^{-1}	1.29 ^{a}	0.98 ^{b}	1.15 ^{a}	2.15 ^{b}
53 kg N ha^{-1}	1.16 ^{b}	1.12 ^{ab}	1.16 ^{a}	2.06 ^{b}

[1]Means followed by different letters at each date were significantly different at P<0.05.

Analysis of primocane leaf samples taken postharvest showed no significant effects in the 2016 season but did show an increase in the N concentration from the high treatment in the 2017 season. No trends in primocane leaves were observed for other nutrients (Supplementary Table D-4).

6.4. Discussion

6.4.1. Red drupelet reversion

The relatively increased levels of RDI in fruit from the high application rate treatment demonstrates that excessive N application can increase the incidence and severity of RDR, however the mechanism behind this increase was unclear, and most of the harvested fruit were still marketable per commercial standards (USDA, 2018).

It should also be noted that the high N application rate used in this trial was significantly higher than that typically recommended commercially. Rates applied commercially vary, as the amount applied relies on results of primocane nutrient analyses, soil tests, observations of annual growth characteristics, and nutrient source (Strik, 2008; Strik and Bryla, 2015). Strik (2008, 2016) reported that an average of 52 kg N ha^{-1} per year was removed in blackberry production systems and recommended application rates in the range of 45 to 84 kg ha^{-1}.

In the 2017 season, an association between harvest dates with fruit skin temperatures exceeding 27 °C (harvests two, five, and six) and higher RDI scores was observed. This trend was not observed in the 2016 season; however, fruit temperatures did not exceed 25 °C at any harvest. This trend is consistent with the previous literature examining harvest conditions and RDR. Yin (2017), McCoy *et al.* (2016), and Chapter 5 demonstrated that time of harvest and the associated fruit temperature is an important factor in the development of RDR. McCoy *et al.* (2016) and Yin (2017) both reported that fruit harvested early in the morning, where average skin temperatures were below 24 °C, had lower RDR incidence than later harvest times where fruit temperatures exceeded 29 °C. Chapter 5 also demonstrated that harvest times associated with fruit temperatures over 25 °C and soil tensions greater than -12 kPa at 30 cm produced fruit with a higher incidence and severity of RDR.

Previous reports have not identified fruit mass as a factor in fruit susceptibility to RDR; however, our data indicate that smaller fruit were less likely to develop the disorder than larger fruit. As fruit size and number of drupelets are related (Strik *et al.*, 1996), this may be due to smaller fruit with lower drupelet numbers having less chance of developing RDR. However, firmness, shape, cellular structure or other physiochemical characteristics may also contribute to this association. There was no clear correlation between firmness and mass, although firmness measurements had a high amount of variation between fruit. This mass-RDR relationship may also contribute to the variability of different blackberry cultivars to RDR development, which has been noted by previous authors (McCoy *et al.*, 2016; Perkins-Veazie and Clark, 2011; Salgado and Clark, 2016; Yin, 2017).

6.4.2. Yield

The data show an association between application rate and increased yield, with the high N treatment producing a significantly higher yield than the low treatment in 2016, and a higher yield than both other treatments in 2017. This was attributable to more fruit in 2016, and both more and larger fruit in 2017 (Figs. 2, and 3). The increase in yield was much larger in 2017, most likely due to the biennial nature of the floricane fruiting blackberries. This is consistent with previous trials (Malik and Archbold, 1991; Naraguma and Clark, 1998b; Strik, 2008), which have generally reported that fertiliser applications of N are primarily used for new growth including primocanes, so a yield response is more likely in the second year. It is unclear what mechanisms may have contributed to the significant yield response in the 2016 season; however, it is possible that the low N treatment may have negatively affected flowering, fruit set or lateral development in spring when the fertiliser regime began. In terms of marketable yield, the actual increase from the higher N treatments in 2017 was likely less significant than the data suggest due to the fruit quality from all treatments towards the end of the 2017 season being poor. This can be observed in the significant decline in firmness (Fig. 4) and sugars (Supplemental Table D-3) in late-season fruit. In 2016, there was a week of severe weather (>32 °C, <35 % relative humidity) around harvest three, which caused the loss of some buds and developing fruit and contributed to the lower total yield in 2016 compared to 2017. The trend for declining average fruit mass over the course of the season, particularly for 2017, is consistent with the previous work of Takeda *et al.* (2003) and Fernandez-Salvador *et al.* (2015) for a number of different blackberry cultivars. Smaller fruit size towards the end of the season is a common occurrence across environments and cultivars, possibly due to carbohydrate competition as supply is directed towards other tissues and plant storage rather than fruit development (Strik, 2008). The average weight (7.8–12.1 g) reported here is higher than that reported previously for 'Ouachita' fruit by Clark and Moore (2005) (4.5–6.8 g), though no previous data exist for the cultivar being grown under tunnels in comparable conditions to the temperate Tasmanian environment.

6.4.3. Firmness

The observed trend for softer fruit towards the end of both seasons is consistent with Fernandez-Salvador *et al.* (2015), who reported that 'Marion', 'Black Diamond', 'Obsidian', and 'Triple Crown' blackberries declined in firmness during the first season and considerably during the second season of a two-year study. The firmness values contained a large amount of deviation between individual fruit within each harvest and treatment, which may reflect the variable nature of blackberry fruit even when harvest methods attempted to select fruit at the same ripeness stage of 'shiny black'.

The Firmtech II machine also had increased occurrences of failure to record a reading for small and/or soft fruit, of which there was a larger amount towards the end of each season. Perkins-Veazie *et al.* (2000a) described similar limitations for using compression measurements on soft fruit after storage, where soft and mushy fruit could not be statistically differentiated from firmer fruit. Blackberries and other small fruits have been assessed for firmness by subjective human assessment, hand-held and benchtop compression or penetration devices (Cahn *et al.*, 1992; Fernandez-Salvador *et al.*, 2015; Perkins-Veazie *et al.*, 2000b; Perkins-Veazie *et al.*, 1997; Salgado and Clark, 2016). Though devices using compression to measure firmness, such as the Firmtech II, have been successfully used previously to assess cultivar differences in some situations (Perkins-Veazie *et al.*, 2000a; Salgado and Clark, 2016), the technique was problematic in this trial. In associated work (Chapter 4), we found that a penetrometer device was able to detect significant differences with less variability than a compression device for 'Ouachita' blackberries subjected to different harvest treatments.

6.3.4. Physiochemical properties

The lower pH of fruit from the low N application rate relative to both other rates for harvest one in 2016, and harvests one, three, and six in 2017 is an association which has been previously reported. Alleyne and Clark (1997) reported lower fruit pH from zero N application compared to 56 and 112 kg N ha^{-1}, and Ali *et al.* (2012) reported lower fruit pH when fertilised with 60 kg N ha^{-1} + 66 kg K ha^{-1} relative to 100 kg N ha^{-1} + 104 kg K ha^{-1}.

It is possible that the small change to fruit pH may contribute to a reduction in stability of the anthocyanins found in the fruit (Fossen *et al.*, 1998; Welch *et al.*, 2008). However, the differences in berry pH between N treatments did not correlate with the harvest dates at which differences in RDI were observed, so it is unlikely that the pH change was linked to any increased susceptibility to RDR. The possible link between N supply and pH may warrant further investigation to examine if the effects may be more significant over a longer trial period, in different environments, and with other cultivars. As well as this, the effect may be more important in the production of blackberries for processing purposes, where juice pH can be an important factor for determining quality (Cahn *et al.*, 1992).

The inverse correlation between monomeric anthocyanins and fruit size indicates that anthocyanin production was consistent and concentration was diluted with increased fruit size, an effect which has been reported previously (Ali, 2012; Anttonen and Karjalainen, 2009). Whilst anthocyanin concentration was not associated with the incidence of RDR, the correlation between fruit size and concentration may be of note to breeders and fruit processors as anthocyanin concentration is a significant factor in fruit appearance and overall fruit quality.

6.3.5. Fruit and plant nutrient concentrations

At three of the six harvest dates at which fruit macronutrient concentrations were analysed, fruit N concentration was highest from the high treatment. This suggests that N fertiliser application rates did influence the amount of N moving into the fruit during the trial, particularly when early in the season. Strik and Vance (2016) and Strik (2008) reported that early in the season, newly acquired N is partitioned primarily to new growth including fruit, primocanes and primocane leaves. Towards the end of growing seasons, new N is stored in roots, crowns and over-wintering primocanes (Malik and Archbold, 1991; Naraguma and Clark, 1998b; Strik and Vance, 2016). Our results are consistent with this – with early season fruit N concentration higher in the high N treatment in both years, and primocane leaf concentration higher in 2017.

The fruit N concentrations of 1.01–1.31 % that were observed (Table 6-2) were slightly lower than previously reported (1.4–1.6 %) for floricane-bearing blackberry cultivars (Strik 2008), though the average fruit mass in this trial was higher than other authors have reported for ‘Ouachita’ blackberries (Clark and Moore 2005) so the lower N concentration may be due to dilution. No conclusive data exist for the impact of fruit N concentration on fruit quality (Strik, 2008), though increased N concentration has been linked to poor postharvest quality in other commodities such as apples (Marcelle, 1995; Raese *et al.*, 2007) and strawberries (Cantliffe *et al.*, 2007).

6.4. Conclusions

Our results indicate that N application rates to 'Ouachita' blackberries grown under tunnels can significantly influence the incidence and severity of RDR, and that fruit mass and harvest date are also significant factors in RDR development. The high N application rate produced fruit with more RDR compared to low and medium rates on five out of 11 harvest dates over a two-year period. Small fruit were much more likely to have no incidence of the disorder, and harvest dates with cooler temperatures generally had a lower incidence. It was hypothesised that elevated N application rates and warmer fruit temperatures made fruit more susceptible to mechanical injury causing cell damage; however, the covariates measured could not confirm this. Whilst the increase in RDI was significant, most fruit harvested from all treatments was still marketable, and thus the commercial impact of the increase was negligible. The high N treatment produced significantly increased yield over the low N treatment due to more fruit in 2016, and over both other rates due to more and larger fruit in 2017. We have demonstrated that RDR incidence and severity is influenced by N fertilisation and environmental conditions, which has important implications for agronomic management for this cultivar. While the establishment of a relationship between plant nutrition and RDR susceptibility is important, more work is needed with a range of cultivars and environments to examine the underlying physiological mechanisms. Alternative firmness analysis techniques, skin textural properties, and cell structural differences should also be considered as areas for future study.

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Chapter 7. Flesh temperature during impact injury and subsequent storage conditions affect the severity of colour change caused by red drupelet reversion in blackberries

This chapter investigates the effect of rapid cooling following impact injury to blackberries. The potential for rapid rates of cooling to exacerbate the disorder has been raised by producers repeatedly and investigated with mixed results by previous authors. In this trial, we investigated rapid versus slow cooling under laboratory conditions.

This chapter has been submitted for publication as a conference paper for the proceedings of the III International Berry Fruit Symposium, 2018, Istanbul, Turkey.

Abstract

Red drupelet reversion (RDR) is a physiological occurrence in blackberries where drupelets revert from black at harvest to red postharvest. The objectives of this trial were to assess the effects of fruit temperature during mechanical injury and temperature changes following injury of blackberries on the subsequent development of RDR.

Individual fruit were subjected to mechanical injury from a steel ball dropped from a height of 25 cm at initial temperatures of 15, 25, and 35 °C. Following injury, fruit were either rapidly cooled to 2 °C in a -24 °C cooler or slowly in a 2 °C cooler. The colour of the impact site and of the undamaged control fruit were measured 24 h and 7 days after the initial impact injury using a colorimeter. Impact injury caused a significant colour difference (ΔE) compared to the control in 95 % of fruit. There were also significant interactions between initial temperatures and cooling rates on the colour of the impact site 24 h and 7 days after treatment. Higher fruit temperatures at the time of mechanical injury and a faster cooling rate post-injury were associated with increased lightness and

chroma. The results confirm that mechanical injury to blackberry fruit leads to RDR, and that the temperature of fruit at the time of injury can influence the severity of RDR.

Keywords: Bruising, impact injury, reversion, CIELAB, storage

7.1. Introduction

Red drupelet reversion (RDR) in blackberries (*Rubus* L. subgenus *Rubus* Watson) is a postharvest physiological disorder causing individual drupelets that are black at harvest to revert to a red colour, usually during postharvest cool storage. The disorder can affect from a single drupelet to over half of the fruit, reducing the visual quality and marketability. Currently, the physiology of the disorder is not well understood. However, with increasing worldwide blackberry production (Strik *et al.*, 2007), there is a growing amount of interest from growers and researchers to understand the underlying mechanisms. Recent research has linked incidence of the disorder to reduced berry firmness (Salgado and Clark, 2016), high rates of nitrogen fertiliser application (Chapter 6), and harvest times associated with high temperatures (Chapter 5; McCoy *et al.*, 2016; Yin, 2017). Chapters 4, 5 and Pérez-Pérez *et al.* (2018) both showed that postharvest development of RDR was associated with mechanical injury incurred during harvest and transport.

Despite these recent findings, a greater understanding of conditions which cause fruit to be more susceptible to developing the disorder is needed to develop management strategies to reduce incidence and severity.

Blackberry producers have colloquially reported that fruit that is rapidly cooled from high field temperatures tends to be more prone to developing RDR than fruit that undergoes a slower cooling process, particularly when forced-air coolers are used to rapidly remove field heat. No studies have examined this experimentally, though fruit temperature at harvest has been shown to be a significant factor in the susceptibility of fruit to developing RDR (Chapter 5; McCoy *et al.*, 2016). McCoy *et al.* (2016) reported no significant trends in the percent of berries with reversion across eight cultivars between storage temperatures of 1 °C and 5 °C, but the author suggested the need for continued research into the interaction of postharvest storage conditions and RDR.

High airflow rates are generally accepted as being best practice to rapidly cool fruit from the field, with the modern fresh-market blackberry industry often using forced-air coolers at 0–2 °C and high relative humidity (Strik *et al.*, 2007). Delays to cooling of as short as one hour have been shown to reduce the percentage of marketable strawberry fruit (Mitcham and Mitchell, 2002), with delays of six hours enough to increase water loss by 50 % (Nunes *et al.*, 1995). No previous reports exist for any potential physiological effects of cooling rates in blackberries, although similar issues have been investigated in other horticultural commodities. DeMartino *et al.* (2002) reported that apricots (*Prunus armeniaca* L.)which had been mechanically damaged and underwent a change of temperature (4 °C to 18 °C and 18 °C to 4 °C) showed increased levels of respiration, ethylene production and bruise response than fruit which was stored at a constant temperature after bruising. Whitelock *et al.* (1994) examined how the thermal and physical properties of peaches (*Prunus persica* L.) affected weight loss and demonstrated that high airflow rates increased weight loss and decreased fruit firmness.

Dehydration during forced-air cooling of lychee (*Litchi chinensis* L.) fruit has been identified as a factor causing cell decompartmentalisation, loss of cell structural integrity and subsequent degradation of anthocyanins from browning reactions (Liang *et al.*, 2013).

The aims of this trial were to assess the effects of fruit temperature during impact damage and the subsequent rate of temperature change on the severity of colour change induced in RDR.

7.2. Materials and methods

7.2.1. Site and field trial design

'Ouachita' blackberries were harvested from a commercial berry farm in Dunorlan, Tasmania, Australia. Canes were on a two-wire trellis system, with 105 m long rows spaced at 2.5 m under 4 m high tunnels covered with high-UV transmittance polythene. Canes were fertilised and irrigated throughout the season as per industry standards (Strik, 2005).

7.2.2. Harvest

Blackberries were harvested on February 12, 2018. Fruit for harvest were selected at random from both sides of each row under a single polytunnel and were otherwise free of pests, pathogens, or physical damage. Fruit were harvested without handling or damaging by using Felco 100 Cut and Hold Secatuers (Felco Australia Pty Ltd, Australia) to cut the pedicel approximately 1 cm above the fruit, and were then gently placed into a cotton wool-lined cell of a plastic seedling tray for transport. Within one hour of harvest, the fruit were transported in a cooler on ice to the Tasmanian Institute of Agriculture (Hobart, Tasmania, Australia) for experimental treatments and analysis.

7.2.3. Treatments and postharvest experimental design

In order to induce RDR, each fruit was bruised with mechanical impact. A 19.2 g stainless steel ball was dropped from a height of 25 cm onto the side of each fruit through a length of plastic tubing with holes drilled in the sides to minimise air resistance.

No discernible bounce back of the ball was observed from any impacts, so it was assumed that the fruit absorbed all the impact force spread over the impact site and the opposing side of the fruit.

A completely randomised factorial design was used with two factors totalling six treatments. Factors were initial fruit temperature at the time of bruising (15, 22, 35 °C ±1 °C), and cooling rate ('fast' or 'slow') following impact. 80 fruit were randomly assigned to each of the bruise temperature treatments. 40 fruit per treatment were bruised and 40 were unbruised controls. Of each group of 40 fruit, 20 were subjected to each cooling rate treatment after the impact injury.

In order to reach the correct treatment temperatures, fruit were placed into temperature-controlled rooms or ovens at 15, 25, and 35 °C, and fruit skin temperature was monitored every five minutes until they reached the target temperatures (±0.5 °C). Fruit were then subjected to impact injury and subsequent storage treatments. Following the impact treatments, fruit were cooled to 3 ± 0.5 °C either rapidly in a -20 °C freezer, or slowly in an ambient 2 °C cool room. During cooling, skin temperature was constantly monitored to make sure that the fruit skin did not pass below 3 °C. The control fruit underwent the same temperature changes as the damaged fruit, but were not damaged as a result. Once the fruit had reached the target temperature for storage (3±0.5 °C), trays were transferred back to commercial storage conditions of 2 °C and 95 % relative humidity for later quality assessment.

7.2.4. Colour change

The impact site on each fruit and a site on the side of each control fruit were assessed for colour change 24 h and 7 days after the initial impact injury. CIELAB colour space values (L*, a*, b*) were measured using a CR-400 colorimeter (Konica Minolta, Australia). Chroma (C*) and hue angle (h^o) were calculated by the formulas:

$$C^* = \sqrt{(a^* \times a^*) + (b^* \times b^*)}$$

$$h^\circ = tg^{-1}(\frac{b^*}{a^*})$$

Mean colour difference (ΔE*) between the control fruit and the impact site of damaged fruit was calculated using the CIEDE2000 colour difference formula (Luo *et al.*, 2001):

$$\Delta E = \sqrt{(\frac{\Delta L^*}{k_L S_L})^2 + (\frac{\Delta \mathrm{C}^*}{k_C S_C})^2 + (\frac{\Delta \mathrm{H}^*}{k_H S_H})^2 + \Delta \mathrm{R}}$$

7.2.5. Statistical analysis

Analysis of variance (ANOVA) was used to examine the effects of initial temperature, cooling rate, and their interactions on CIELAB colour variables. Tukey's Honest Significant Difference (HSD) post-hoc tests were used to determine differences in the means of the CIELAB colour values between treatments at significance levels of 0.05. R version 3.4.3 (R Core Team 2017) was used for all statistical analysis.

7.3. Results and discussion

7.3.1. Inducing RDR

The site of the impact injury had significant colour change in over 95 % of fruit, while less than 5 % of control fruit was affected by any colour change. The extreme care taken when harvesting and transporting the fruit ensured that the only mechanical injury to the fruit was that induced by the experimental treatments. In the fruit which did not have any colour change at the impact site, the individual berry was often leaking heavily or was mouldy at the 7-day reading which made colorimeter measurement difficult. The high rate of success inducing RDR with mechanical injury is in agreement with reports by Pérez-Pérez *et al.* (2018) and Chapter 4, that suggested that cell damage from mechanical injuries is likely to be a key physiological mechanism of the disorder.

No treatment had any significant effects or interaction for any CIELAB colour variables in undamaged fruit, and no variables of undamaged fruit were significantly different between 24 h and seven days (Fig. 7-1). Most of the colour differential between red and black flesh was contributed to by lower chroma values and slightly lower lightness, with the values for hue angle being highly variable between samples of black fruit.

7.3.2. Colour change

On average, the impact site was significantly lighter (higher L* value) and brighter (higher chroma value) than black fruit both 24 h and seven days after the initial injury, as well as having a lower hue angle (°hue) seven days after the initial injury (Tables 1, 2). Despite this, the change of colour was easily discernible to the eye and represents a significant loss in marketability of the fruit.

Table 7-1. Mean colour values from each cooling rate, initial bruise temperature treatment and mean black values 24 h after treatments.

Cooling Rate	Initial Temp	L*	a*	b*	Chroma	Hue°
Slow	15 °C	19.7±1.4 bc	9.5±2.0 a	3.5±0.9 a	10.2±2.1 a	20.3±3.3
Slow	25 °C	19.2±1.4 c	10.6±3.2 ab	3.7±1.3 a	11.2±3.5 ab	19.3±1.9
Slow	35 °C	20.5±1.6 abc	9.6±2.5 a	3.4±1.0 a	10.2 ±2.7 a	19.6±2.7
Fast	15 °C	19.3±2.7 c	8.7±3.5 a	3.3±1.3 a	9.3±3.8 a	21.1±3.3
Fast	25 °C	21.7±1.2 a	10.9±2.6 ab	3.9±1.2 ab	11.6±2.8 ab	19.6±2.1
Fast	35 °C	21.2±1.6 ab	12.7±2.6 b	5.1±1.1 b	13.7±2.8 b	21.8±1.2
Control		17.3±1.7 c	1.8±0.4 c	0.7±0.5 c	2.0±1.6 c	21±9.7

Means followed by different letters in each column were significantly different at the $P<0.05$ level.

After seven days, fruit from the fast-cooling treatment continued to lighten and increase in chroma slightly, but the control and fruit from other treatments colour remained largely unchanged. This indicates that most of the colour change associated with RDR induced by impact injury occurred within 24 h of the initial cell damage. For future studies, this may reduce the need for extended trial periods.

Table 7-2. Mean colour values from each cooling rate, initial bruise temperature treatment and mean black values seven days after treatments.

Cooling Rate	Initial Temp	L*	a*	b*	Chroma	Hue°
Slow	15 °C	21.2±1.6 ab	10.5±2.7 a	3.9±1.0 a	11.2±2.9 a	19.0±1.3
Slow	25 °C	20.9±0.9 ab	10.5±1.7 a	3.9±0.8 a	11.2±1.8 ab	19.2±1.6
Slow	35 °C	21.4±2.0 ab	10.7±3.2 a	4.2±1.0 a	11.5±3.3 a	20.3±2.8
Fast	15 °C	20.4±1.3 b	10.1±3.4 a	4.1±1.5 a	10.9±3.7 a	20.5±2.2
Fast	25 °C	22.2±1.7 a	12.4±4.0 ab	4.7±1.5 ab	13.3±4.3 b	19.6±1.4
Fast	35 °C	22.3±1.4 a	13.9±1.6 b	5.7±0.6 b	15.0±1.7 b	20.7±1.0
Control		18.0±1.3 c	1.6±0.5 c	0.9±0.3 c	1.9±0.5 c	28±7.0

Means followed by different letters in each column were significantly different at the $P<0.05$ level.

The temperature at the time of bruising, the cooling rate, and the interaction between these factors produced significant effects on the lightness and chroma values ($P<0.05$) of the impact site. After both 24 h and seven days, flesh that was injured at warmer temperatures had higher lightness and chroma values than fruit injured at lower temperatures. A faster cooling rate post-injury also resulted in increased values for lightness and chroma in fruit which was bruised at 25 and 35 °C, but there was no difference in any variables between cooling rates for fruit bruised at 15 °C.

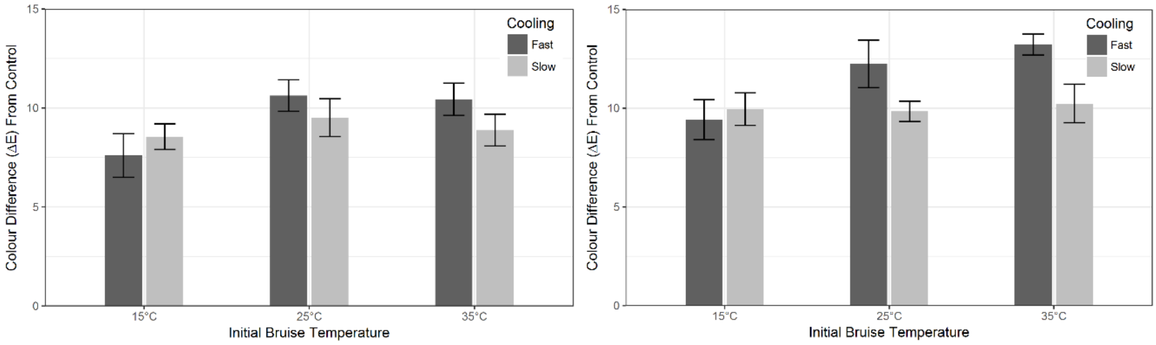

Fig. 7-1. Mean colour difference (ΔE) between control fruit and the impact site of each treatment 24 h (left) and 7 days (right) after impact

The differences in the colour space measurements resulted in significant effects for the cooling rate and initial temperature on colour difference (ΔE*) 24 h after impact, and for the cooling rate seven days after impact (Fig. 7-1.). The differences between the control fruit and impact site for 'fast' cooled fruit was more significant after seven days due to slight changes in the colour profile at the impact site.

It has been theorised and reported by previous authors that physical injury can affect the thermal properties of fruit tissue through accelerating or reducing transpiration rate, density and conductivity, and consequently heat production/dissipation within the bruised tissue (Segovia-Bravo *et al.*, 2011; Van Linden *et al.*, 2003). Varith (2001) reported that bruised tissue in apples had a 9–26 % higher thermal conductivity and 5 % higher density, resulting in a 4–20 % higher thermal diffusivity. This led to the development of a method for the detection of bruises in apples using thermal imaging to identify areas of flesh with varying levels of thermal diffusivity which changed temperature at different rates (Varith *et al.*, 2003). If mechanical injury to blackberries results in similar physiological changes then this may result in the damaged flesh being more prone to moisture loss or further cellular damage, brought on or exacerbated by rapid temperature changes. This may potentially be a contributing mechanism to the results observed in this trial, though further experimental evidence is needed to identify the underlying physiology involved.

The methodology of assessing RDR in blackberries has not been widely discussed within the previous literature. Previous trials have involved some level of human subjectivity when measuring RDR, and severity of colour change has not been discussed in depth (McCoy *et al.*, 2016; Salgado and Clark, 2016). The results in this trial suggest that severity of colour change is not equal between affected drupelets or fruit, and is a variable which should be considered in future trials. Worthington *et al.* (2017) described the preliminary development of a system which used digital image analysis to rapidly and accurately count the incidence of reversion across several cultivars. This use of imaging software to assess RDR is promising, as with further development it could potentially remove the subjectivity and allow for a standardisation of methodology across future trials.

7.4. Conclusions

The results of this trial indicate that there is an association between mechanical injury, temperature during injury, and subsequent storage conditions that can influence the severity of colour change associated with RDR. Warm fruit temperatures during impact bruising and subsequent storage conditions experiencing rapid temperature changes resulted in more severe colour change in damaged flesh. Fruit in these conditions had higher lightness and chroma values as measured by a colorimeter, which resulted in a greater colour differential from the control fruit.

In order to reduce the severity and possibly the incidence of RDR, it is recommended that mechanical damage to fruit should be avoided, particularly at temperatures exceeding 25 °C, which has implications for management practices such as the time to harvest on warm days. This is in agreement with previous findings (Chapters 5, 6; McCoy *et al.*, 2016; Yin 2017). However, it has been noted that cultivar and harvest time have a significant interactive effect on RDR susceptibility (Chapter 2; Yin, 2017; Lawrence and Melgar, 2018). When fruit is mechanically injured, reducing the rate of cooling may potentially reduce the severity of colour change, though producers should consider this method with caution and consider any implications of a delay in cooling on other postharvest parameters.

This research may lead to improved management practices that reduce the postharvest incidence of RDR. Further research is required to investigate the physiological mechanism responsible for the differences in the severity of colour change observed.

7.5. Acknowledgements

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Chapter 8. General discussion and conclusions

This chapter summarises the body of work presented in this thesis, discusses the relevance of this work to the blackberry industry, and addresses the four key research goals outlined on pages xi-xii. General conclusions, key findings, and recommendations for future research are presented.

8.1. General discussion

This thesis delivers numerous findings that advance the current knowledge surrounding RDR, may impact management strategies for commercial blackberry producers, and be of relevance to future studies investigating this topic. The research chapters established the underlying physiochemical processes involved in the development of RDR and then identified several environmental and management factors contributing to RDR susceptibility and development. The implications of these findings offer potential management options to reduce the incidence and severity of RDR in commercial blackberry production, as well as stimulate further study in this area. This chapter addresses the four key research goals, summarises the findings of the project, and discusses the implications of this research for the blackberry industry and future research.

8.2. Research goals

8.2.1. To identify and quantify the physiochemical changes occurring in drupelets affected by RDR

The physiochemical changes associated with RDR are reported in Chapter 4. This chapter established that the colour change characterising RDR is induced by a degradation of anthocyanin pigments in affected tissue, which is accompanied by cellular disruption, loss of membrane integrity, decompartmentalisation, increased intracellular spaces, loss of firmness and reduced pH. The physiochemical changes were consistent with symptoms of mechanical injury to affected drupelets, which is in agreement with the recent similar findings and suggestions by Pérez-Pérez *et al.* (2018) and Salgado (2015). Further work is required to identify the exact mechanism of anthocyanin degradation, though the observed structural and chemical changes are consistent with decompartmentalisation of anthocyanins from cell vacuoles and their subsequent enzymatic degradation, which has been widely reported for many anthocyanin-containing foods (Castañeda-Ovando *et al.*, 2009; Lee and Wicker, 1991; Welch *et al.*, 2008).

Anthocyanin degradation from mechanical injury in fruit is generally associated with brown colour development due to the formation of brown polymers during the enzymatic degradation reaction. No authors to date have noted any visible browning associated with RDR, though this may be due to the anthocyanin concentration in reverted drupelets still being in the range of 700–1100 mg kg^{-1} FW, and the pH remaining less than 3.5. Given that the browning index in fruit juices from anthocyanin degradation has been demonstrated to be pH dependant (Dorris *et al.*, 2018; Jiang *et al.*, 2019), and that anthocyanin decolourisation at higher pH has been shown to contribute to tissue browning in fruit (Underhill and Critchley, 1994), these conditions may mask the formation of brown pigments. Thus, the reduction in anthocyanin concentration and the slight pH reduction favours the red colour development associated with RDR.

The data from Chapter 4 also indicate that in drupelets affected by RDR, anthocyanin species containing disaccharides or acylated sugar moieties are not degraded as readily as those containing monosaccharides and non-acylated sugars. This is consistent with previous reports for anthocyanin stability (Cevallos-Casals and Cisneros-Zevallos, 2004; Welch *et al.*, 2008), and may have further implications for blackberry researchers and breeders. It has been colloquially suggested that the colour change associated with RDR varies in severity and shade between cultivars; something that variation of species within the anthocyanin profile may influence. Whilst more data are needed to support this suggestion, an increase of acylated anthocyanin species within the profile of cultivars may contribute to observable differences in the severity of colour change. This finding warrants further investigation in a range of cultivars grown in disparate environmental conditions to examine the effects of anthocyanin profile on RDR severity.

Chapter 4 established that RDR results in a significant softening of affected drupelets, most likely caused by the observed cellular structural damage and loss of turgor. This suggests that RDR may play a larger role in the postharvest quality and shelf-life of blackberries than has been established to date, given that fruit softening is a factor in mould development (Perkins-Veazie *et al.*, 1997). Furthermore, fruit firmness can be a key indicator of freshness and quality to consumers (Redgwell and Fischer, 2002; Ross *et al.*, 2009), so decreasing RDR incidence will further impact quality perception. Fruit that were harvested without handling, such as those described in Chapter 5, displayed similar firmness values after 14 days storage to fruit that were hand-harvested after 7 d in storage (data not shown). If a causative link exists between RDR and mould development, our findings and future work to reduce RDR incidence may further contribute to increased fruit quality, shelf-life and profitability of blackberry production.

The examination of the underlying physiochemical changes occurring in drupelets affected by RDR produced important findings to guide the direction of the following research chapters and will continue to contribute to the further study of RDR.

8.2.2. To identify any physical or environmental factors involved in expression of RDR

Chapters 5 and 7 demonstrated that damage incurred by handling or impacts to fruit are a factor in RDR development, which was consistent with the conclusion from Chapter 4: mechanical injury was associated with RDR development. This finding now supports previous suggestions that mechanical injury may lead to RDR (Salgado 2015) with experimental evidence.

The data presented in Chapter 5 also indicate that conditions promoting fruit skin temperatures which exceed 23 °C were associated with increased RDR incidence and severity. These results support similar conclusions shown by McCoy *et al.* (2016) and Yin (2017): harvest times with warm temperatures may exacerbate the disorder, though Lawrence and Melgar (2018) demonstrated that this effect can vary with genotype and other environmental factors. Despite this, it can be concluded that in order to reduce RDR incidence in commercial production, growers should aim to utilise harvest techniques and conditions that minimise fruit temperatures during harvest. In the Tasmanian climate, harvest times prior to midday offer these conditions, which provides growers with sufficient hours to complete daily harvests. However, this will vary with location.

The potential confounding effects of soil water status and VPD should be further investigated across a larger number of cultivars in order to make additional management recommendations relevant to a variety of production zones.

8.2.3. To identify plant nutrition that may be contributing to an increase in RDR.

The establishment of an interaction between nutrient fertiliser application rates and RDR incidence, as presented in Chapter 6, is a key finding of this project. This is the first reported link between fertiliser application rates and RDR incidence, so this work may encourage broader research into the physiological mechanisms behind this association as well as further investigations into nutrient-fruit quality interactions. The effects of the N application rate and fruit N concentration on fruit yield and quality in blackberries is inconclusive in previous literature, where studies have reported conflicting or inconsistent results, possibly due to variations in cultivars, agronomic practices, soil, and the environment (Strik, 2008). Hence, whilst the findings from Chapter 6 are important in establishing the potential for nutrient fertiliser application rates to affect RDR incidence, no definitive management recommendations for individual production systems can be made from this study alone. This paper is relevant to the wider literature addressing nutrient-fruit quality relationships in *Rubus*, however, and may explain some grower observations of high N rates leading to increased RDR in commercial settings.

It was hypothesised that any interaction between N fertiliser rate and RDR incidence may emanate from changes in fruit firmness. No significant effect on firmness was observed in the data; however, as discussed in Chapter 6, compression firmness testing can produce inconsistent results, particularly for soft fruit. We recommend that penetration tests, or other alternative methodologies for assessing fruit firmness, should be explored in future studies that can examine the effects of nutrient fertiliser application rates on RDR and fruit quality in blackberries.

8.2.4. To identify and develop potential pre- or postharvest techniques to reduce the incidence of RDR.

Potential management techniques for reducing the incidence and severity of RDR were identified in Chapters 5, 6, and 7. Chapter 5 established that mechanical injury incurred during harvest and transport of fruit is an underlying factor in the development of RDR, and that fruit temperatures exceeding 23 °C during handling significantly increase incidence and severity. Aside from aiming to avoid harvest times associated with these conditions, the use of structures such as shade cloth to reduce heat exposure, or manipulation of cane architecture to shade the fruit are options that should be explored. Before these recommendations are put into practice, further study should be undertaken to fully understand any other effects of reducing light exposure on plant and fruit quality.

It is currently common practice in the Australian blackberry industry to pick blackberries into buckets and then transfer the fruit into punnets when the buckets are full. This reduces labour costs, which in Australia are a significant portion of production cost, though the practice is likely a major source of compression injury to fruit. In Chapter 5, 85 % of fruit harvested using this technique contained at least one reverted drupelet after 24 h in cold storage, compared to just 6 % of fruit which was not handled during harvest. Whilst harvesting without handling is an impractical technique for commercial settings, as it would increase labour costs prohibitively, these data do demonstrate that even light handling can significantly reduce the postharvest quality of blackberries. This finding highlights the importance of reducing handling during harvesting, as well as proper picker training to reduce compression injury to fruit. No studies have investigated any finer points of harvest techniques such as ‘twisting’ versus ‘pulling’, and while it is common for growers to recommend that pickers use a ‘twist’ technique, the seasonal and untrained nature of the workforce may make this difficult to ensure.

Chapter 2 highlights the significant variation in RDR expression between cultivars, and it can be concluded that cultivar selection for the specific growing environment is vital for reducing losses to RDR. Given the difficulty of importing new cultivars into Australia due to rigid biosecurity restrictions, depending on RDR-resistant cultivars is not an easy, short-term solution. However, breeding or importing cultivars with low susceptibility in Australian conditions should be considered for the longer-term management of RDR.

8.3. Other implications and findings arising from this project

Due to the lack of a published comprehensive review of the literature detailing the extent of the current knowledge of RDR, the literature review contained in Chapter 2 of this thesis is of importance to further investigations in this field. The rapid expansion of the worldwide blackberry industry over the last two decades has not been fully matched with an increase in study into the fruit’s physiology, highlighted by the lack of published data on RDR as well as other physiological disorders and plant-soil interactions. In recent years, this project and other concurrent studies have resulted in a significant growth in knowledge surrounding the genotypic variance, physiochemical mechanisms, and environmental influences on RDR expression. Chapter 2 consolidates the information generated from the previous sporadic studies and current work into a comprehensive article summarising the available data on RDR. It is intended that this chapter will be of interest to both academic and commercial parties, as well as promoting a deeper understanding of this complex and commercially important disorder.

The published data contained in this thesis are solely from experiments carried out with the cultivar ‘Ouachita’. This was necessary due to the relatively small-scale blackberry production industry in Tasmania, limiting the available experimental sites, as well as to allow for a broader range of experiments without replicating for multiple cultivars. Whilst this presents some limitations given the genotypic variance in RDR susceptibility and development demonstrated throughout the wider literature and discussed in Chapter 2, there are obvious trends in the physiochemical and structural observations between our data and other published studies, as demonstrated in Chapters 2 and 4. This suggests that the underlying physiological mechanisms involved in the development of and susceptibility to the disorder remain consistent across cultivars. Specifically, reports of anthocyanin reduction by 40–60 %, loss of cellular structural integrity, and an association with mechanical injury are consistent across cultivars and climatically disparate environments.

8.4. Future research direction

RDR is an issue of growing importance to blackberry producers and researchers, as evidenced by the increasing number of research projects concurrent to this one investigating various aspects of the disorder in recent years (Lawrence and Melgar, 2018; McCoy *et al.*, 2016; Pérez-Pérez *et al.*, 2018; Worthington *et al.*, 2017; Yin, 2017). This increase has seen substantial growth in the understanding of the underlying physiological mechanisms and causes of RDR. Despite this, considerable knowledge gaps still exist in this area of research.

Further study to clarify any underlying physiological reasons for the genotypic variance in incidence and severity will be of interest to breeders and growers in order to develop cultivars with low susceptibility to RDR. As well as this, a better understanding of what physiological characteristics provide resistance to RDR may further confirm or clarify our conclusions as to the major factors causing RDR expression.

There are a growing number of studies investigating the effects of preharvest environmental factors on RDR expression, such as those shown in Chapter 5. While some consistency has been reported across disparate environments for the effects of temperature during harvest on RDR incidence, the effects appear to vary with genotype and are potentially confounded by other climatic variables.

Additional data for a range of commercially important cultivars may clarify these conclusions, though care should be taken to assess a broad range of climatic variables to minimise any bias in results.

Nutritional links to RDR should be further investigated. This thesis established that increased N rates can influence RDR incidence; however, the underlying causes behind this remain unclear. This thesis offers substantial opportunities to continue and broaden this area of research to fully understand the influence of nutrient fertiliser application rates on RDR expression.

As discussed in Chapters 2 and 6, inconsistencies currently exist in the reported techniques used to assess the incidence and severity of RDR. In order to better enable future researchers and industry to be able to compare rates of the disorder across studies and environments, work should be done to develop a standard management technique for sampling the incidence and severity of RDR in practical settings. While counting the total number of affected drupelets per fruit and/or attempting to classify levels of severity in affected drupelets is time-consuming, a technique such as this offers the most in-depth data about severity of the disorder. Additionally, the incidence of affected drupelets per fruit at several different levels (e.g. 1+, 3+, or 5+ drupelets) can be reported in order to allow for comparison with most other studies. Alternatively, the use of imaging software to digitally assess RDR incidence may offer rapid, accurate, and unbiased evaluation, though such techniques may not be widely available or practical in all situations.

8.5. Conclusions

The findings presented in this thesis have relevance to commercial blackberry producers, retailers, and breeders, as well as implications for future research into both RDR and broader postharvest quality of blackberry fruit. The results from Chapter 4 show that the physiochemical symptoms associated with RDR are consistent with mechanical injury to fruit, resulting in cell decompartmentalisation and the subsequent degradation of anthocyanin pigments.

Chapter 5 demonstrated that handling of fruit is strongly associated with RDR development, and that environmental conditions resulting in fruit skin temperatures exceeding 23 °C during handling can significantly exacerbate the incidence and severity of the disorder. These findings offer further support to the conclusions from Chapter 4, and strongly implicate cell disruption as a major mechanism involved in RDR development. Chapter 7 then demonstrated that storage conditions following mechanical injury can influence the severity of colour change associated with RDR. This suggests that postharvest storage may be able to be manipulated in order to reduce the severity of the disorder, which offers opportunity for further study.

The results from Chapter 6 show that incidence of RDR can be influenced by the N application rate. This effect varied with harvest date but was significant in six out of 11 harvests over the two-year trial. This finding offers some explanation for previous anecdotal observations and offers opportunity for further research into the effects of plant nutrition on RDR and broader blackberry fruit quality.

The data presented in this thesis establish some key mechanisms and causes of RDR. This research has highlighted the importance of environmental factors, fruit handling practices, agronomic management and postharvest factors in RDR development. These findings will contribute to the development of management techniques and future studies incorporating a range of blackberry cultivars and growing environments.

8.6. Summary of key findings

- RDR in blackberries is associated with cellular disruption, loss of membrane integrity and decompartmentalisation in affected drupelets. These processes lead to the degradation of anthocyanin pigments and the resultant colour change associated with the disorder.
- Mechanical injury incurred during handling and transport of fruit is strongly associated with the development of RDR.

- Environmental conditions causing fruit temperatures to exceed 23 °C during harvest appear to significantly exacerbate the degree of structural damage incurred by handling.
- Excessive N application during fruit development and harvest may be associated with increased incidence of RDR. However, rates typically applied in commercial production did not affect the incidence or severity of RDR.
- Inter- and intra-seasonal variation in RDR incidence and severity is likely caused by variation in environmental conditions at individual harvest dates.
- Rapid cooling following mechanical injury may exacerbate the severity of colour change.

8.7. Summary of recommendations for managing RDR in commercial blackberry production

- The development of cultivars with low susceptibility to RDR should be pursued.
- Harvest techniques should be optimised to reduce double and rough handling of fruit.
- Correctly training harvest workers should be a high priority for producers in order to reduce mechanical injuries incurred during harvest.
- Harvesting timing should avoid the handling of blackberries at extreme temperatures. This includes harvesting during the early morning or evening and avoiding harvesting on extremely warm days.
- Cane and field management should be designed around reducing the field heat that fruit are exposed to. Cane architecture to encourage fruit shading, the use of shade cloth, or shading structures should be considered.
- Punnet design and postharvest technologies to reduce mechanical injury to fruit should be explored. Unnecessary fruit-on-fruit contact could be reduced through using punnets which contain only one layer of fruit. With the emergence of larger-fruiting cultivars, the common punnet design may need to be adjusted to better suit these varieties.

- Agronomic management techniques should be investigated further to fully understand the nutrient-fruit quality relationships for specific cultivars and environments. Any links between agronomic management and fruit firmness should be explored.
- Postharvest storage including temperature during handling and rapid temperature changes can influence the severity of RDR, though reducing cooling rate should be thoroughly evaluated for further effects on shelf-life.
- The effect of temperature on the amount of vibration damage incurred during transport of fruit should be investigated.

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Chapter 9. Appendices

Appendix A: Fact sheets arising from this project

1. Edgley, M, Close, D.C., Measham, P.F., 2016. Managing Red Drupelet Disorder, Tasmanian Institute of Agriculture – Fact sheet

2. Edgley, M, Close, D.C., Measham, P.F., 2017. Managing Red Drupelet Disorder', Tasmanian Institute of Agriculture – Fact sheet

3. Edgley, M, Close, D.C., Measham, P.F., 2017. Managing Red Drupelet Disorder', Tasmanian Institute of Agriculture – Fact sheet (2)

4. Edgley, M, Close, D.C., Measham, P.F., 2018. Managing Red Drupelet Disorder', Tasmanian Institute of Agriculture – Fact sheet

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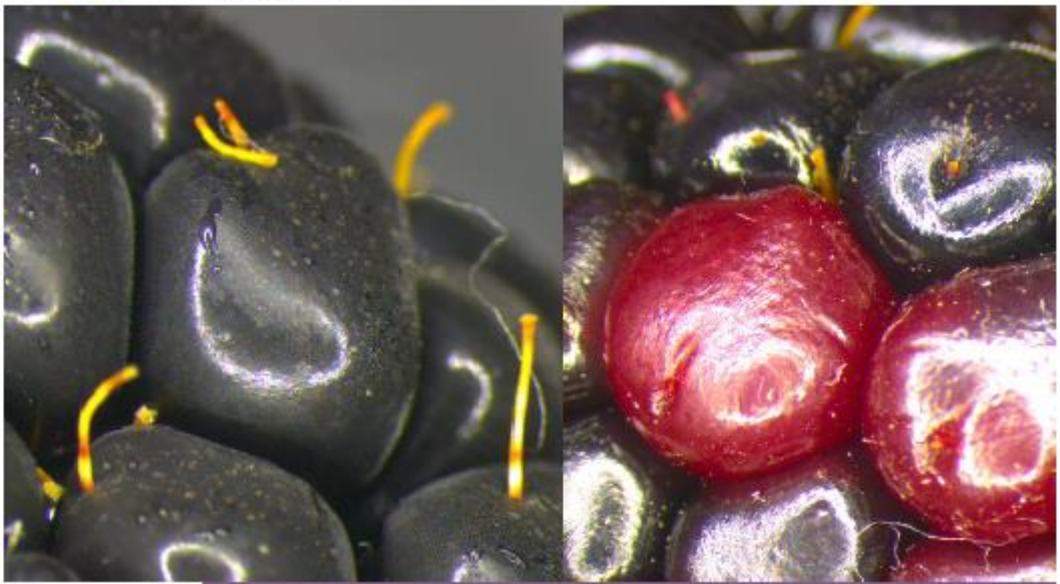

Perennial Horticulture Fact Sheet

Managing Red Drupelet Disorder

Key Points

- A three year project, commenced in August 2015
- The project aims to understand the physiology behind drupelet reversion and develop management techniques to reduce the incidence of the disorder
- Research will assess both pre and post-harvest contributing factors
- Preliminary results indicate that fruit cooled at a slower rate is less prone tothe disorder, and the disorder is much more prevalent when fruit is damaged at very cold or very warm temperatures.
- Excess nitrogen during harvest has been shown to produce higher rates of the disorder

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Introduction

Red drupelet disorder (RDD), sometimes referred to as drupelet reversion or reddening, is a physiological disorder of blackberry fruit. Individual drupelets that appear uniform in colour with the rest of the fruit at harvest revert to a red colour following cool storage. Although there are a number of other causes for blackberries to change colour including UV damage, freeze damage, leakage, and insect damage, RDD is thought to be independent of these. Drupelet disorder can affect up to 50% of a crop and is one of the least understood postharvest problems in blackberry fruit production.

More Information

Red drupelet disorder can affect up to 50% of a crop , with anywhere from single drupelets to almost whole fruit being prone. Because the disorder generally appears following harvest and storage, the financial loss from severely affected fruit can be significant.

Affected fruit does not taste any different, however the disorder is off-putting to consumers. Reverted drupelets also appear to be more prone to other damage from leakage and attack by pathogens.

The disorder is heavily influenced by cultivar, with moderate environmental and seasonal variability.

Image 1: Blackberry fruit affected by red drupelet disorder

Underlying Physiology

The underlying physiology of the disorder is not fully understood, but research is underway in this area. The end result of the disorder is a loss of around 50% of the anthocyanin pigment that gives blackberries their dark colour (cyanidin-3-glucoside). It is not clear what reaction is causing this loss. Enzyme activity, pH changes, and physical rupture of the cells are known to cause pigment destruction in other fruit and it is likely that one or more of these play a role in red drupelet disorder.

Year one trials

The first year of field and lab trials were based around inducing the disorder and understanding what contributing factors may be causing high rates of the disorder. This involved:

- Evaluating the impact of storage conditions and physical damage on expression of the disorder
- Investigating the role of nutrition management in red drupelet disorder
- Understanding the underlying physiology of what is happening inside the cells during loss of colour

Preliminary Results

Preliminary results indicate significant factors in drupelet reversion are fruit temperature at harvest and during storage when fruit is most likely to be physically damaged. Fruit that is cooled at a slower rate is less prone to the disorder, and the disorder is much more prevalent when fruit is damaged at very cold or very warm temperatures. As well as this, excess nitrogen application during summer was shown to increase incidence of the disorder.

Image 2: Control fruit (A), fruit physically damaged at 2°C (B), and fruit damaged at 10°C (C)

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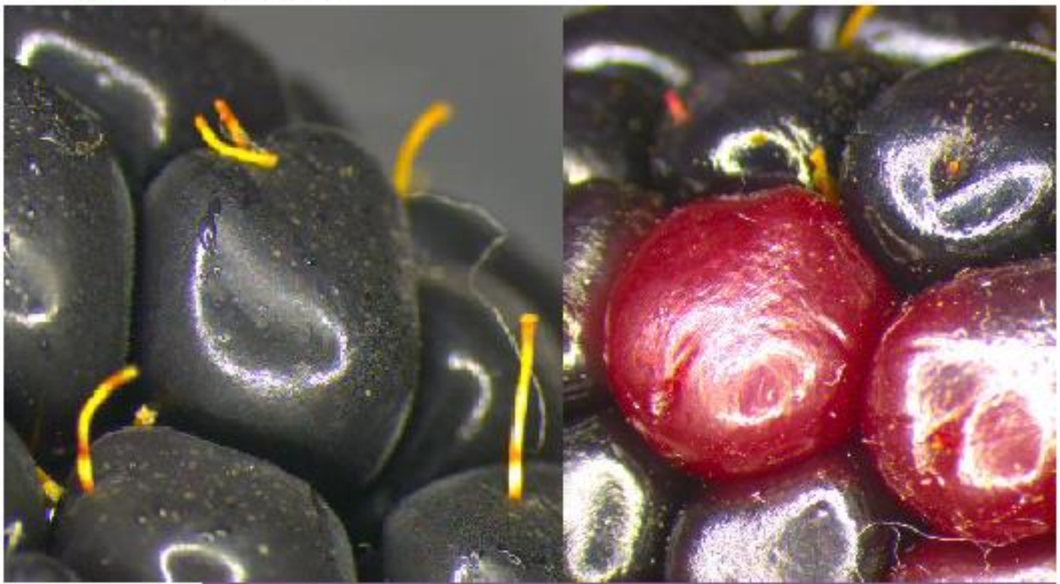

Perennial Horticulture Fact Sheet

Managing Red Drupelet Disorder

Key Points

- Physical damage during harvest and shipping is the main cause of red drupelet disorder; but fruit is more prone under certain conditions
- High nitrogen fertigation during harvest can significantly increase the amount of fruit with red drupelet disorder post-harvest
- Fruit core temperatures exceeding 23C at harvest significantly increase the amount of red drupelet post-harvest
- Harvest times, techniques, and shipping conditions can be manipulated to reduce incidence of red drupelet disorder
- A step-cooling process reducing the rate of cooling post-harvest has been effective in reducing incidence of the disorder

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Introduction

Red drupelet disorder (RDD), sometimes referred to as drupelet reversion or reddening, is a physiological disorder of blackberry fruit. Individual drupelets that appear uniform in colour with the rest of the fruit at harvest revert to a red colour following cool storage. Although there are a number of other causes for blackberries to change colour including UV damage, freeze damage, leakage, and insect damage, RDD is thought to be independent of these. Drupelet disorder can affect up to 50% of a crop and is one of the least understood postharvest problems in blackberry fruit production.

Underlying Physiology

The underlying mechanism responsible for the disorder is a degradation of the anthocyanin pigments which give blackberry fruit their colour. This happens when the cells of the fruit are damaged at harvest or during transport, and is exacerbated by certain environmental conditions such as rapid changes of temperature after damage.

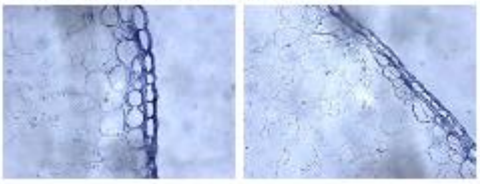

Image 1: Healthy blackberry cells (left) compared to damaged cells in drupelets affected by red drupelet disorder (right)

Harvest and Post-harvest Conditions

Environmental conditions such as temperature, humidity, and plant water status at harvest. Fruit which has a higher core temperature at harvest is significantly more prone to developing red drupelet post-harvest.

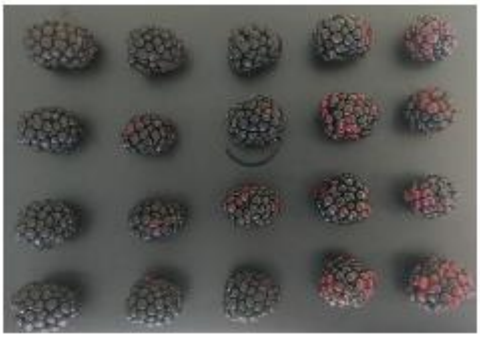

Image 2: Fruit harvested at increasing core temperatures (left to right)

Rate of Cooling

The rate at which fruit is cooled post-harvest has also been shown to play a role in the expression of the disorder. Fruit which is cooled at a slower rate in a 'step-cooling' process had significantly lower incidence of red drupelet in one trial. It is thought that rapid temperature change following physical damage to the fruit can worsen the structural damage.

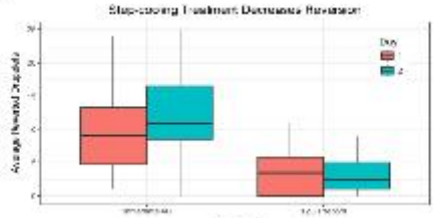

Figure 1: Results from a two day trial comparing storage techniques

Nitrogen Fertigation

The project has included a two-year field trial looking into the effects of nitrogen fertigation rates on post-harvest expression of the disorder. The results of this study include:

- High nitrogen application rates during harvest produced higher rates of red drupelet disorder
- Higher nitrogen rates also produced larger fruit for parts of the season, and higher overall yields

Ongoing Work

Work is ongoing to shed further light on the physiology of the disorder, as well as assessing potential management techniques to reduce incidence.

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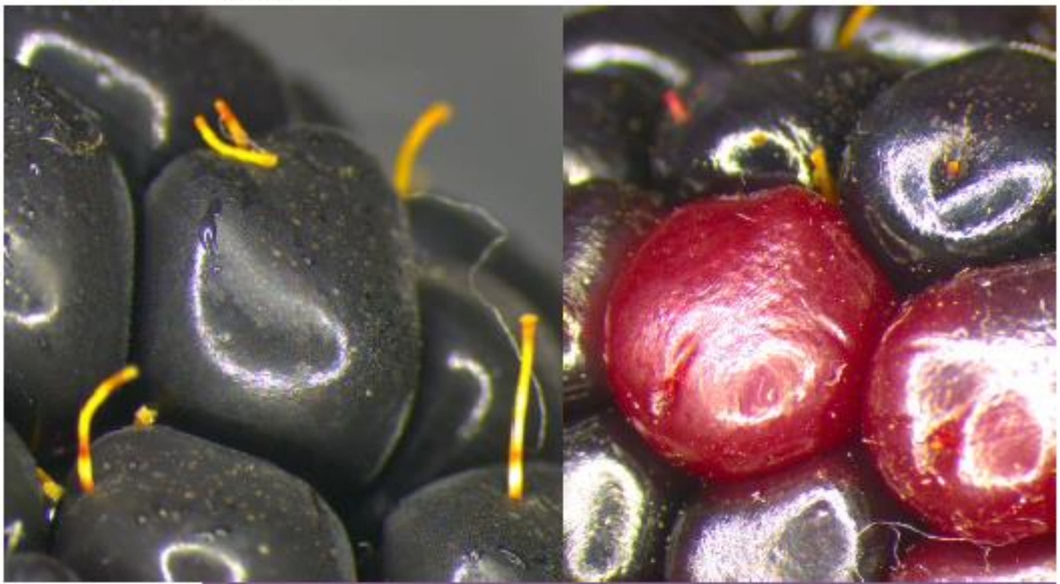

Perennial Horticulture Fact Sheet

Key Points

- Mechanical injury during harvest and shipping is the main cause of red drupelet reversion; but fruit is more prone under certain conditions
- High nitrogen fertigation during harvest can significantly increase the amount of fruit with red drupelet reversion post-harvest
- Fruit flesh temperatures exceeding 23°C at harvest significantly increase the amount of red drupelet post-harvest
- Harvest times, techniques, and shipping conditions can be manipulated to reduce incidence of reversion
- A step-cooling process reducing the rate of cooling post-harvest has been effective in reducing incidence of the disorder

Managing Red Drupelet Reversion

Introduction

Red drupelet reversion (RDR), sometimes referred to as drupelet reversion or reddening, is a physiological disorder of blackberry fruit. Individual drupelets that appear uniform in colour with the rest of the fruit at harvest revert to a red colour following cool storage. Although there are a number of other causes for blackberries to change colour including UV damage, freeze damage, leakage, and insect damage, RDR is thought to be independent of these. The disorder can affect up to 50% of a crop and is one of the least understood postharvest problems in blackberry fruit production.

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Underlying Physiology

The underlying mechanism responsible for the disorder is a degradation of the anthocyanin pigments which give blackberry fruit their colour. This happens when the cells of the fruit are damaged at harvest or during transport, and is exacerbated by certain environmental conditions such as rapid changes of temperature after damage.

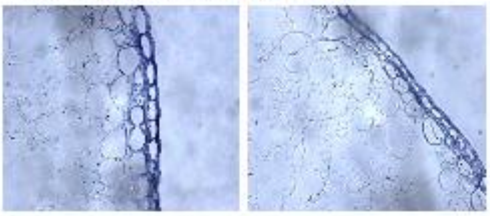

Image 1: Healthy blackberry cells (left) compared to damaged cells in drupelets affected by red

Harvest and Post-harvest Conditions

Environmental conditions such as temperature, humidity, and plant water status at harvest may influence expression of RDR. Fruit which has a higher skin temperature at harvest is significantly more prone to developing red drupelet post-harvest.

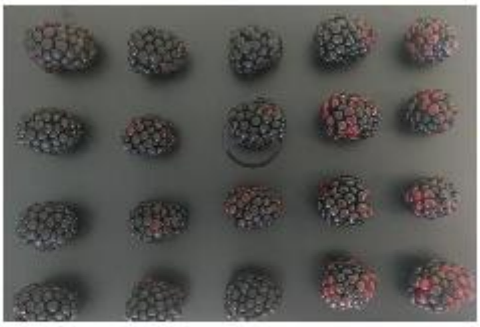

Image 2: Fruit harvested at increasing skin temperatures (left to right)

Rate of Cooling

The rate at which fruit is cooled post-harvest has also been shown to play a role in the expression of the disorder. In one trial, fruit which was cooled extremely quickly after being damaged had more severe colour change than fruit which was cooled at a slower rate.

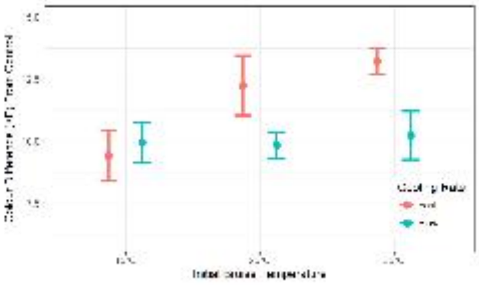

Figure 1: Results comparing bruise temperature

Nitrogen Fertigation

The project has included a two-year field trial looking into the effects of nitrogen fertigation rates on post-harvest expression of the disorder. The results of this study include:

- High nitrogen application rates during harvest produced higher rates of red drupelet reversion
- Higher nitrogen rates also produced larger fruit for parts of the season, and higher overall yields

Ongoing Work

Further analysis and results from the project are ongoing and will be available later this year.

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Hort Innovation
Strategic levy investment

RASPBERRY AND BLACKBERRY FUND

This project has been funded by Hort Innovation, using the raspberry and blackberry research and development levy and contributions from the Australian Government. Hort Innovation is the grower owned, not-for-profit research and development corporation for Australian horticulture

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Perennial Horticulture Fact Sheet

Key Points

- Physical damage during harvest and shipping is the main cause of red drupelet disorder; but fruit is more prone under certain conditions
- High nitrogen fertigation during harvest can significantly increase the amount of fruit with red drupelet disorder post-harvest
- Fruit core temperatures exceeding 23C at harvest significantly increase the amount of red drupelet post-harvest
- Harvest times, techniques, and shipping conditions can be manipulated to reduce incidence of red drupelet disorder
- A step-cooling process reducing the rate of cooling post-harvest has been effective in reducing incidence of the disorder

Managing Red Drupelet Disorder

Red drupelet disorder (RDD), sometimes referred to as drupelet reversion or reddening, is a physiological disorder of blackberry fruit. Individual drupelets that appear uniform in colour with the rest of the fruit at harvest revert to a red colour following cool storage. Although there are a number of other causes for blackberries to change colour including UV damage, freeze damage, leakage, and insect damage, RDD is thought to be independent of these. Drupelet disorder can affect up to 50% of a crop and is one of the least understood postharvest problems in blackberry fruit production.

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TIA is a joint venture of the University of Tasmania and the Tasmanian Government

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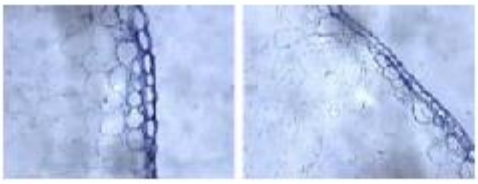

Image 1: Healthy blackberry cells (left) compared to damaged cells in drupelets affected by red drupelet disorder (right)

Rate of Cooling

The rate at which fruit is cooled post-harvest has also been shown to play a role in the expression of the disorder. Fruit which is cooled at a slower rate in a 'step-cooling' process had significantly lower incidence of red drupelet in one trial. It is thought that rapid temperature change following physical damage to the fruit can worsen the structural damage.

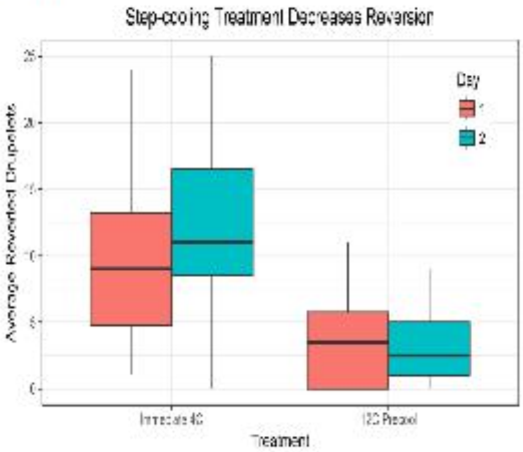

Figure 1: Results from a two day trial comparing storage techniques

Harvest and Post-harvest Conditions

Environmental conditions such as temperature, humidity, and plant water status at harvest can influence red drupelet development. Fruit which has a higher core temperature at harvest is significantly more prone to developing red drupelet post-harvest.

Image 2: Fruit harvested at increasing core temperatures (left to right)

Nitrogen Fertigation

The project has included a two-year field trial looking into the effects of nitrogen fertigation rates on post-harvest expression of the disorder. The results of this study include:

- High nitrogen application rates during harvest produced higher rates of red drupelet disorder
- Higher nitrogen rates also produced larger fruit for parts of the season, and higher overall yields.

Ongoing Work

Work is ongoing to shed further light on the physiology of the disorder, as well as assessing potential management techniques to reduce incidence.

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Appendix B: Additional material pertaining to Chapter 4

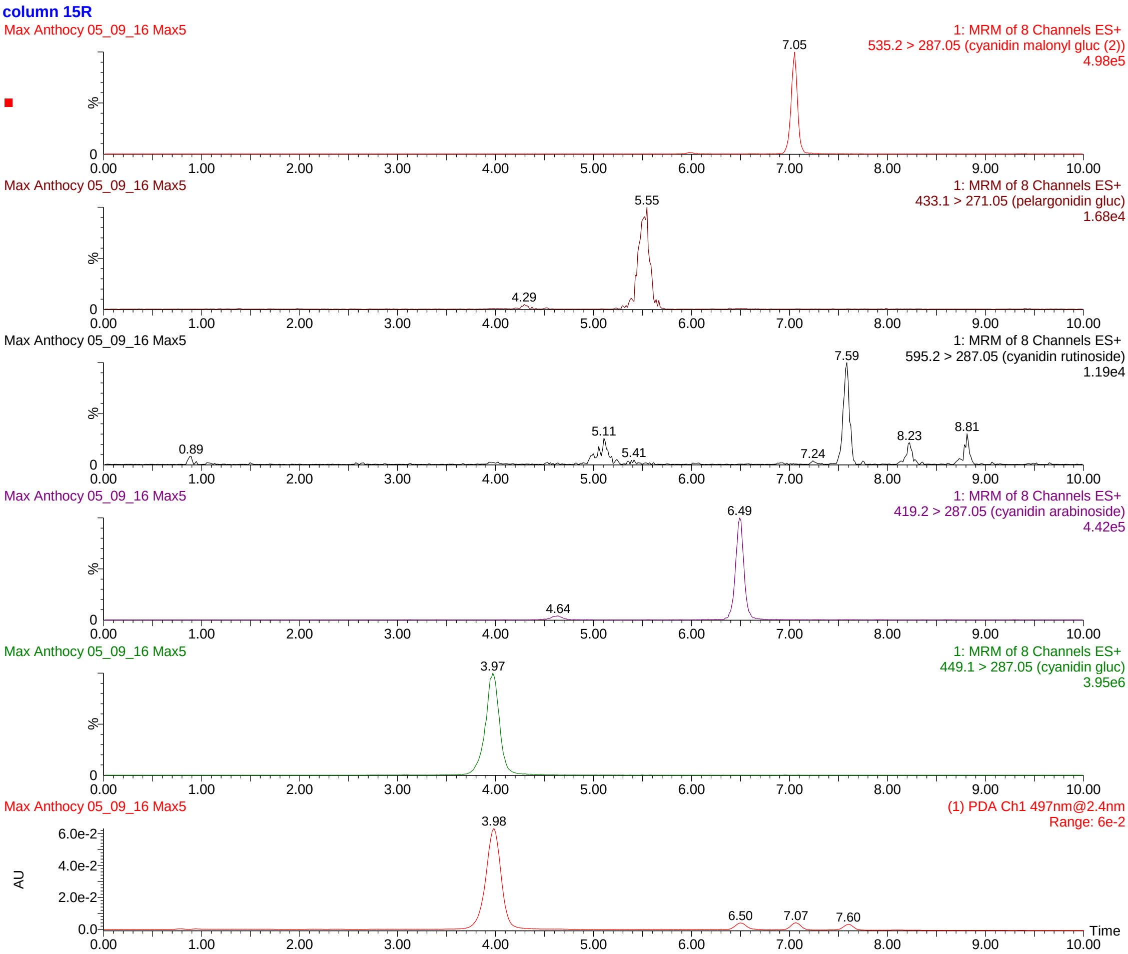


Fig. B-1. Chromatogram of anthocyanins identified in ‘Ouachita’ samples

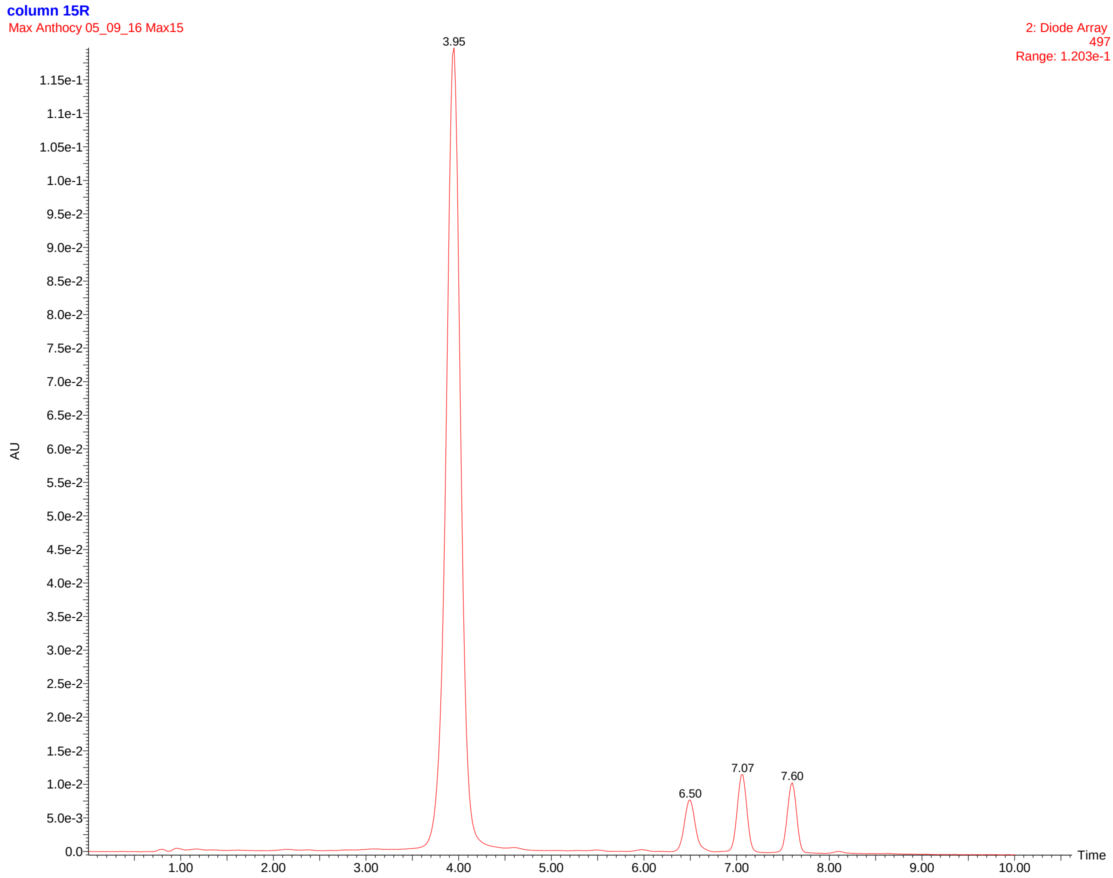


Fig. B-2. Chromatogram of minor anthocyanins identified in 'Ouachita' samples

Table B-1. Retention times of each anthocyanin extracted via UPLC.

Anthocyanin:	**Retention time (min)**
Cyanidin-3-glucoside	3.9
Cyanidin-3-arabinoside	4.4
Cyanidin-3-rutinoside	5.0
Pelargonidin-3-glucoside	5.3
Cyanidin-3-(3''-malonylglucoside)	5.8
Cyanidin-3-xyloside	6.5
Cyanidin-3-(6''-malonylglucoside)	7.0
Pelargonidin-3-glucoside	7.5

Appendix C: Additional material pertaining to Chapter 5

Fig. C-1. Fruit from harvest treatment 1 (bottom) and harvest treatment 2 (top).

Fig. C-2. Fruit from harvest treatment 2 (undamaged) 24 days after harvest. Little to no mould was observed and fruit retained high firmness.

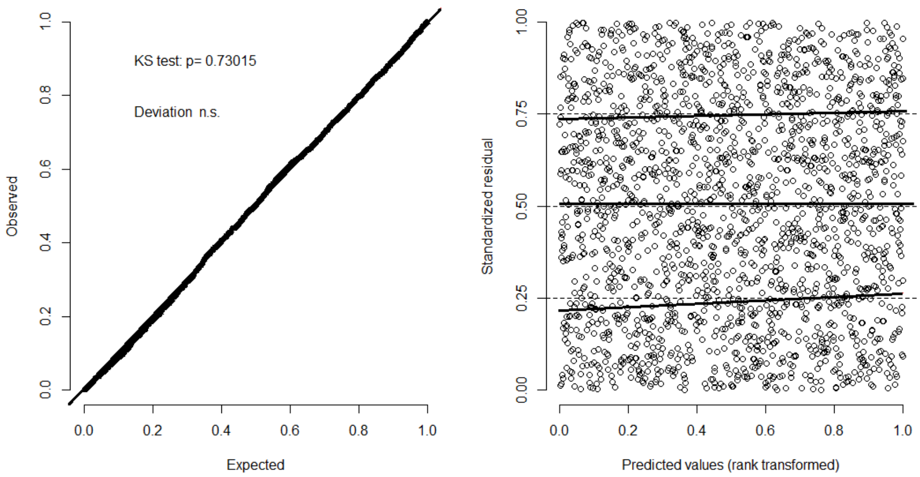
Appendix D: Additional material pertaining to Chapter 6

D.1 Supplementary figures and tables

Supplementary table D-1. Model coefficient estimates and significance.

Effect	Estimate	Std. Error	Z Value	Pr(>|z|)
Nitrogen Treatment	3.21	1.44	2.23	0.03
Harvest Date	-0.10	0.08	-1.44	<0.01
Mass	-0.67	0.23	-2.98	<0.01

[1]Model: $RDI = N\ Treatment + Harvest\ Date + Mass$
[2]Log-likelihood: -3597 on 29 df



Supplementary fig. D-1. Residual diagnosis for the best-fitting zero-inflated negative binomial model. QQ plot with KS test statistic is shown on the left, and residuals versus predicted values on the right.

Supplementary table D-2. Means of titratable acidity, total soluble sugars, sugar:acid ratio, and monomeric anthocyanin (ACN) concentrations in 2016.

Harvest	N Treatment	TA (% citric acid)	TSS (°brix)	TSS:TA	ACN (mg 100g^{-1} fresh weight)
	High	0.90 ^{abcde}	11.3 ^{a}	12.7 ^{abcdef}	58.2 ^{c}
1	Medium	0.84 ^{abcde}	11.5 ^{a}	13.8 ^{abcd}	63.3 ^{bc}
	Low	0.77 ^{bcde}	11.3 ^{a}	15.0 ^{abc}	66.2 ^{bc}
	High	1.05 ^{ab}	10 ^{abc}	9.6 ^{ef}	70.0 ^{abc}
2	Medium	0.98 a^{bcde}	9.5 ^{bc}	9.9 ^{ef}	72.7 ^{abc}
	Low	1.02 ^{ab}	10.3 ^{abc}	10.1 ^{def}	77.6 ^{abc}
	High	1.01 ^{abc}	10.3 ^{abc}	10.4 ^{cdef}	67.8 ^{bc}
3	Medium	1.09 a	10.8 ^{abc}	9.9 ^{ef}	65.0 ^{bc}
	Low	0.99 ^{abc}	11.0 ^{ab}	11.2 ^{bcdef}	70.0 ^{abc}
	High	1.06 ^{ab}	9.2 c	8.7 ^{f}	83.6 ^{ab}

4	Medium	0.99 ^{abc}	9.5 ^{bc}	9.6 ^{ef}	67.9 ^{bc}
	Low	1.00 ^{abc}	9.1 c	9.2 ^{ef}	73.9 ^{abc}
	High	0.69 ^{de}	10.5 ^{abc}	15.4 ^{ab}	81.3 ^{abc}
5	Medium	0.72 ^{cde}	10.7 ^{abc}	14.9 ^{abcd}	80.8 ^{abc}
	Low	0.67 ^{e}	10.1 ^{abc}	16.1 ^{a}	92.5 ^{a}

[1]Means followed by different letters in each column were significantly different at P<0.05.

Supplementary table D-3. Means of pH, TA, TSS, TSS:TA, and ACN concentrations in 2017.

Harvest	N Treatment	TA (% citric acid)	TSS (°brix)	TSS:TA	ACN (mg 100g^{-1} fresh weight)
	High	0.94 ^{a}	12.3 ^{abc}	13.3 ^{ab}	79.2 ^{abc}
1	Medium	1.00 ^{a}	12.0 ^{abc}	11.9 ^{ab}	82.7 ^{a}
	Low	0.93 ^{a}	12.5 ^{abc}	14.0 ^{ab}	81.2 ^{ab}
	High	0.85 ^{a}	13.0 ^{a}	15.5 ^{a}	63.2 ^{abcd}
2	Medium	0.85 ^{a}	12.6 ^{ab}	14.9 ^{a}	63.9 ^{abcd}
	Low	0.77 ^{a}	12.9 ^{a}	16.7 ^{a}	65.4 ^{abcd}
	High	0.95 ^{a}	11.9 ^{abc}	12.6 ^{ab}	38.9 ^{cd}
3	Medium	1.02 ^{a}	12.0 ^{abc}	11.8 ^{ab}	41.0 ^{bcd}
	Low	0.91 ^{a}	12.1 ^{abc}	13.5 ^{ab}	30.9 ^{d}
	High	0.92 ^{a}	10.1 ^{bcd}	11.4 ^{ab}	45.8 ^{bcd}
4	Medium	0.99 ^{a}	10.8 ^{abcd}	11.0 ^{b}	63.8 ^{abc}
	Low	1.06 ^{a}	11.8 ^{abc}	11.1 ^{b}	41.6 ^{bcd}
	High	0.88 ^{a}	11.0 ^{abcd}	12.7 ^{ab}	47.5 ^{abcd}
5	Medium	0.89 ^{a}	10.5 ^{abcd}	11.8 ^{ab}	78.5 ^{abc}
	Low	0.92 ^{a}	10.7 ^{abcd}	11.7 ^{ab}	55.9 ^{abcd}
	High	0.99 ^{a}	9.1 ^{d}	9.2 ^{b}	63.5 ^{abcd}
6	Medium	0.88 ^{a}	9.8 ^{cd}	11.2 ^{b}	45.9 ^{abcd}
	Low	0.93 ^{a}	10.1 ^{bcd}	11.0 ^{b}	54.6 ^{abcd}

[1]Means followed by different letters in each column were significantly different at P<0.05.

Supplementary table D-4. Mean macronutrient concentration in fruit over the course of each season.

Harvest	Treatment	P (%)	K (%)	Ca (%)
2016				
	212 kg N ha^{-1}	0.17	1.10	0.22
1	106 kg N ha^{-1}	0.16	1.10	0.20
	53 kg N ha^{-1}	0.15	0.97	0.16
	212 kg N ha^{-1}	0.15	1.08	0.18
3	106 kg N ha^{-1}	0.16	1.22	0.20
	53 kg N ha^{-1}	0.15	1.06	0.20
	212 kg N ha^{-1}	0.15	0.99	0.21
5	106 kg N ha^{-1}	0.15	1.08	0.19
	53 kg N ha^{-1}	0.16	1.02	0.18
2017				
	212 kg N ha^{-1}	0.15	1.18	0.16
1	106 kg N ha^{-1}	0.14	1.04	0.19

	53 kg N ha^{-1}	0.15	1.14	0.17
3	212 kg N ha^{-1}	0.15	1.15	0.22
	106 kg N ha^{-1}	0.13	0.90	0.17
	53 kg N ha^{-1}	0.14	0.97	0.19
5	212 kg N ha^{-1}	0.14	0.97	0.20
	106 kg N ha^{-1}	0.13	0.93	0.18
	53 kg N ha^{-1}	0.14	1.04	0.23

[1]Means followed by different letters in each column and year were significantly different at P<0.05.

Supplementary table D-5. Phosphorus (P), potassium (K), and calcium (Ca) concentrations of primocane leaf samples taken two weeks postharvest.

Treatment	**P (%)**	**K (%)**	**Ca (%)**
	2016		
212 kg N ha^{-1}	0.17 [a]	1.77 [a]	NA *
106 kg N ha^{-1}	0.19 [a]	1.82 [a]	NA *
53 kg N ha^{-1}	0.19 [a]	1.78 [a]	NA *
	2017		
212 kg N ha^{-1}	0.14 [b]	1.05 [b]	1.28
106 kg N ha^{-1}	0.15 [b]	1.02 [b]	0.93
53 kg N ha^{-1}	0.13 [b]	0.82 [b]	1.23

1Means followed by different letters in each column and year were significantly different at P<0.05.
2 Analysis for Ca concentration was not available for the 2016 season.

D.2. Acta Horticulturae article

The following research article was published in Acta Horticulturae as a refereed conference paper. The article was written after the first year of the two-year nitrogen experiment. Chapter 6 supersedes this article; thus it is included as an appendix and not a stand-alone chapter.

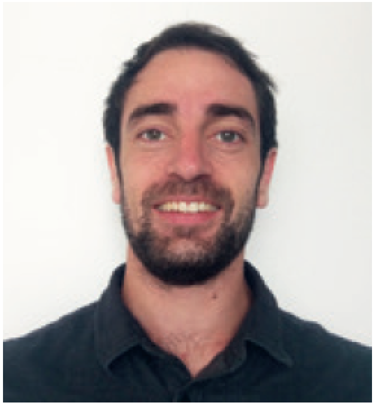
Edgley, M., Close, D.C. and Measham, P.F. (2018). The effects of N fertiliser application rates on red drupelet disorder (reversion) in 'Ouachita' thornless blackberries grown under tunnels. Acta Hortic. 1205, 885-890. DOI: 10.17660/ActaHortic.2018.1205.113

It is: Edgley, M., Close, D. C., Measham, P. F., 2018. The effects of N fertiliser application rates on red drupelet disorder (reversion) in 'Ouachita' thornless blackberries grown under tunnels. Acta horticulturae, 1205, 885-890

D.3. Chronica Horticulturae article

The following news article was published in Chronica Horticulturae (Vol. 57, no. 2, pp. 11), summarising the Acta Horticulturae article (Appendix D.2.).

The effects of nitrogen fertiliser application rates on red drupelet disorder (reversion) in 'Ouachita' thornless blackberries grown under tunnels



› Max Edgley

This project investigated the effects that different rates of nitrogen fertigation had on red drupelet disorder expression in commercial blackberries. Red drupelet disorder (sometimes known as red drupelet reversion or reddening) is a postharvest physiological disorder that causes fruit, which is black at harvest, to revert to a red colour following cold storage. This causes a mottled appearance on the fruit that is off-putting to the consumer and can render fruit unmarketable in some cases, causing significant financial loss to producers. In severe cases, the disorder can affect over half the crop. There is currently very little knowledge surrounding causes and contributing factors to the disorder, with no standard management practices available to reduce incidence. One factor that has previously been suggested as a contributor to high rates of the disorder is excess nitrogen fertilisation close to harvest.

Results showed that higher nitrogen fertigation rates prior to and during harvest, to canes grown under high tunnels, significantly increased rates of red drupelet disorder in fruit. The highest nitrogen application rate used in the study resulted in a 56% increase in expression of red drupelet compared with the lowest application rate throughout the course of the harvest season. This effect was highest at the beginning of the harvest season, at which time red drupelet incidence was highest, and declined as the harvest season progressed. Higher nitrogen rates also increased the total yield, but did not affect berry weight, pH, sugar content, or total acidity. This research shows that a link exists between nitrogen fertigation rates and red drupelet disorder expression, although further work is ongoing to investigate the underlying reason for this increase. These findings have the potential to guide industry standards to reduce incidence of the disorder in commercially produced blackberries.

Max Edgley won an ISHS student award for the best oral presentation at the I International Symposium on Protected Cultivation in Tropical and Temperate Climates & X International Symposium on Protected Cultivation in Mild Winter Climates in Australia in November 2016.

› Contact

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